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SEGREGATION MAMMARY CANCER TO NO MAMMARY CANCER IN THE MARSH ALBINO STRAIN OF MICE [1]

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INTRODUCTION

Since the first report of its originator (*1*), strain 3 of the Marsh albino mouse stock has given various incidences of mammary tumor. Numerous investigators, to whom breeding colonies of the stock have been sent, have obtained varying percentages of tumors in the colonies that they have raised. In its home laboratory, this strain has shown a constantly decreasing rate of incidence of mammary carcinoma.

In 1938 one of us (*2*) recorded an explanation of this progressive decrease in tumor incidence, attributing the falling off in rate to the segregation and expansion, within the home colony, of a family or families, none of the members of which developed this type of neoplasm. Unfortunately, at the time of this report, none of the descendants of the noncancer family was extant.

In an effort to determine whether or not this type of segregation is characteristic of the Marsh strain, a system of single-pair matings was followed as a means of obtaining a family that developed tumors in percentages comparable with those first reported for the stock. Such a family was later expanded in a study of the possible recurrence of segregation to noncancer. The present paper reports such a recurrence.

MATERIALS AND METHODS

Strain 3, as developed by Marsh, was started from a single pair of mice obtained from the well-known Lathrop-Loeb colony. The two original animals were probably related, in fact they were presumably the product of several generations of inbreeding. Nothing definite is known of them, however, except that there was a high incidence of mammary cancer in their ancestry.

The progeny of the original pair obtained by Marsh were mated among themselves; but brother-to-sister matings, after the first filial generation, were studiously avoided until 32 generations of cousin matings had been obtained. Following these 32 generations of cousin matings, brother-to-sister mating was begun and has been followed to the present time.

During the course of this experiment, the animals were fed Purina Fox Chow and water. Both food and water were available to them at all times.

First Expansion of the Stock

In the twenty-first generation of brother-to-sister mating (F_{53}), the stock was narrowed to a single pair. Because of this narrowing, the first family of noncancer segregates was lost.

In the fifty-eighth generation (F_{58}), the stock was narrowed to seven pairs. All these (families A to G) were descendants of the single pair in F_{53}. The expansion of these families represents the efforts to obtain a strain which produced cancer in the percentage reported by Marsh. The system of expansion is illustrated in pedigree chart 1 (family F). The distribution of cancer occurrence and death in these seven families is given in table 1.

Second Expansion of the Stock

Since the percentage of tumors obtained in the first expansion of strain 3 fell short of the percentage reported for the original stock, one pair in the sixty-eighth generation of family F (female 10960 × male 10959) was selected as the initial pair in a new expansion. This pair was chosen because the incidence of mammary cancer in their immediate ancestry had the characteristics of a high-cancer line. In 10 generations, 75 to 90 percent of the females in this line of descent developed mammary carcinoma, thus approximating the incidence of those reported by Marsh. This pair was the product of 32 generations of cousin matings followed by 36 generations of brother-to-sister matings. They may be considered to be closely inbred (*3*).

[1] Received for publication November 14, 1946.

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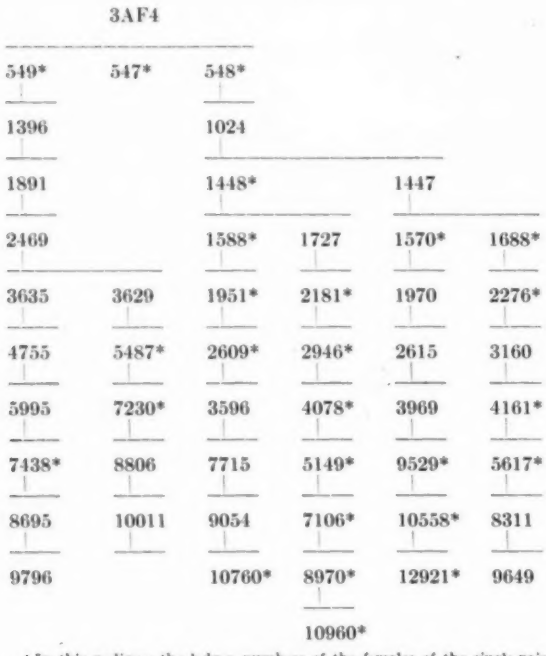
PEDIGREE[1] CHART 1.—Family F, showing system of breeding used in the first expansion of the stock.



[1] In this pedigree the ledger numbers of the females of the single-pair brother-to-sister matings are shown. Those females that developed mammary carcinoma are starred (*).

TABLE 1.—*Cancer incidence in first expansion of stock of Marsh albino breeding females (fifty-eighth to seventieth generation*[1]

Age groups of mice (in days)	Mice with mammary cancer dying	Mice with no mammary cancer dying	Total mice dying	Mice with mammary cancer, this age or later	Mice alive at beginning of period	Percentage of cancer
	Number	*Number*	*Number*	*Number*	*Number*	
150	1	7	8	171	362	47.23
180	1	3	4	170	354	48.02
210	2	5	7	169	350	48.29
240	7	8	15	167	343	48.68
270	6	2	8	160	328	48.78
300	9	14	23	154	320	48.12
330	17	13	30	145	297	48.82
360	15	9	24	128	267	47.94
390	24	13	37	113	243	46.61
420	15	20	35	89	206	43.20
450	14	18	32	74	171	43.27
480	18	18	36	60	139	43.17
510	10	21	31	42	103	40.78
540	9	12	21	32	72	44.44
570	7	5	12	23	51	45.10
600	8	12	20	16	39	41.03
630	4	7	11	8	19	42.05
660	2	3	5	4	8	50.00
690	1	1	2	2	3	66.66
720						
750	1		1	1	1	100.00

[1] Mean age cancer, 438 days.

When female 10960 was 111 days old, she gave birth to her second litter, five males and four females. She nursed all nine for 30 days. At this age, the young were weaned, and five of them (one male and four females) were established as a breeding pen:

$$\text{Females}\begin{cases}12733\\12734\\12735\\12736\end{cases} \times \text{male } 12732$$

EVIDENCE OF SEGREGATION

It was in this litter that segregation apparently took place. In the expansion of this family, mammary cancer appeared in each generation, among the descendants of females 12733, 12734, and 12735. No mammary tumors have been observed among the descendants of female 12736.

At the time of writing, records are available on 15 generations of the descendants of female 12736. No mammary tumors have been observed among the 351 breeding females in this line.

PERCENTAGE OF MAMMARY CANCER AMONG THE DESCENDANTS OF FEMALE 10960

The occurrence of mammary tumor among the descendants of female 10960 is given in table 2.[2] A comparison of the percentages listed in column 7 of this table with the corresponding column in table 1 shows that the production of neoplasms in the second expansion of the stock is appreciably lower than that in the first expansion.

If the descendants of the three cancer-producing females only are tabulated (table 2), it becomes apparent that the percentages of tumor observed in the cancer-producing strains of the second expansion are lower than those found in the first. Apparently only part of the differences between the percentages of tables 1 and 2 may be attributed to the weighing influence of the noncancerous descendants of female 12736.

In summarizing this much of the data, it may be concluded that female 10960 had the following characteristics: (1) With the possible exception of a mutation or mutations, she was in all probability homozygous in all the loci of her chromosomes; (2) the physiology produced by her chromosomes was receptive to the mammary-tumor inciter which she herself received because she herself developed mammary cancer; and (3) she had the ability to transmit the mammary-tumor inciter to her young, as is evidenced by the appearance of cancer in her daughters.

[2] The animals listed in table 2 include only those whose life histories were completed prior to 1943.

The segregation observed in the young of female 10960 may therefore be explained on one or both of two premises: (1) A genetic mutation which produced in daughter 12736 a physiology not receptive to the stimulation of the mammary-tumor inciter received; and (2) a change in physiology that propagated the inciter in varying amounts or potence.

TABLE 2.—*Descendants of female 10960*

DESCENDANTS INCLUDING DAUGHTER 12736[1]

Age group of mice (in days)	Mice with mammary cancer dying	Mice with no mammary cancer dying	Total mice dying	Mice with mammary cancer this age or later	Mice alive at beginning of period	Percentage of cancer
	Number	*Number*	*Number*	*Number*	*Number*	
180	1	7	8	65	235	27.66
210	4	2	6	64	227	28.19
240	3	4	7	60	221	27.15
270	4	7	11	57	214	26.64
300	5	6	11	53	203	26.11
330	4	14	18	48	193	25.11
360	2	6	8	44	174	25.29
390	3	6	9	42	166	25.30
420	5	10	15	39	157	24.84
450	5	11	16	34	142	23.94
480	5	11	16	29	126	23.02
510	10	15	25	24	110	21.82
540	4	14	18	14	85	16.47
570	4	9	13	10	67	14.93
600	5	14	19	6	54	11.11
630	----	15	15	----	35	----
660	----	6	6	----	20	----
690	----	5	5	----	14	----
720	1	5	6	1	9	----
750	----	3	3	----	3	----
DAUGHTERS 12733, 12734, AND 12735[2]						
180	1	5	6	65	168	38.69
210	4	1	5	64	162	39.06
240	3	3	6	60	157	38.21
270	4	4	8	57	151	37.75
300	5	4	9	53	143	37.06
330	4	12	16	48	134	35.82
390	3	5	8	42	111	37.84
420	5	7	12	39	103	37.86
450	5	6	11	34	91	37.36
480	5	8	13	29	80	36.25
510	10	9	19	24	67	35.82
540	4	9	13	14	48	29.17
570	4	5	9	10	35	28.57
600	5	8	13	6	26	23.08
630	----	9	9	----	13	----
660	----	1	1	----	4	----
690	----	1	1	----	3	----
720	1	----	1	----	2	50.00
750	----	1	1	----	1	----

[1] The descendants of female 10960, including daughter 12736, totaled 248 mice, with a mean cancer age of 439 days.
[2] The mean cancer age was 439 days.

One characteristic of genetic mutations is that they happen relatively infrequently. In this instance, variations were observed in the four animals of one litter indicating some cause other than mutation. The possibility of changes in the ability of these animals to propagate the mammary-tumor inciter in unequal concentrations is less well understood.

Because of the fortuitous nature of the development of pedigree patterns and the chance that changes in the ability to propagate the inciter might go unnoticed, re-examination of the pedigrees obtained in the first expansion of the stock may furnish additional evidence that segregations similar to the segregation observed in the young of female 10960 may have occurred previously.

RE-EXAMINATION OF THE FAMILIES A TO G IN THE FIRST EXPANSION

As mentioned previously, seven single-pair matings were used as the progenitors in the first expansion of the stock. In each of these families, there are lines of descent in which mammary tumors appear frequently; in each there are other lines in which these neoplasms appear intermittently, and still others in which the appearance of mammary cancer is the exception.

Family A (pedigree chart 2) illustrates the two extremes. It has a high-cancer branch and a low-cancer branch. In 10 generations of the low-cancer branch, mammary tumors were observed in 3 of the 29 animals tested. In the high-cancer branch, mammary tumor was observed in 8 of 10 generations. Ten of the seventeen animals tested produced tumors. In none of the lines of descent in the first expansion, however, is the segregation as clear-cut and distinct as it is in the second expansion among the progeny of female 12736.

FURTHER EVIDENCE OF INCITER VARIATION

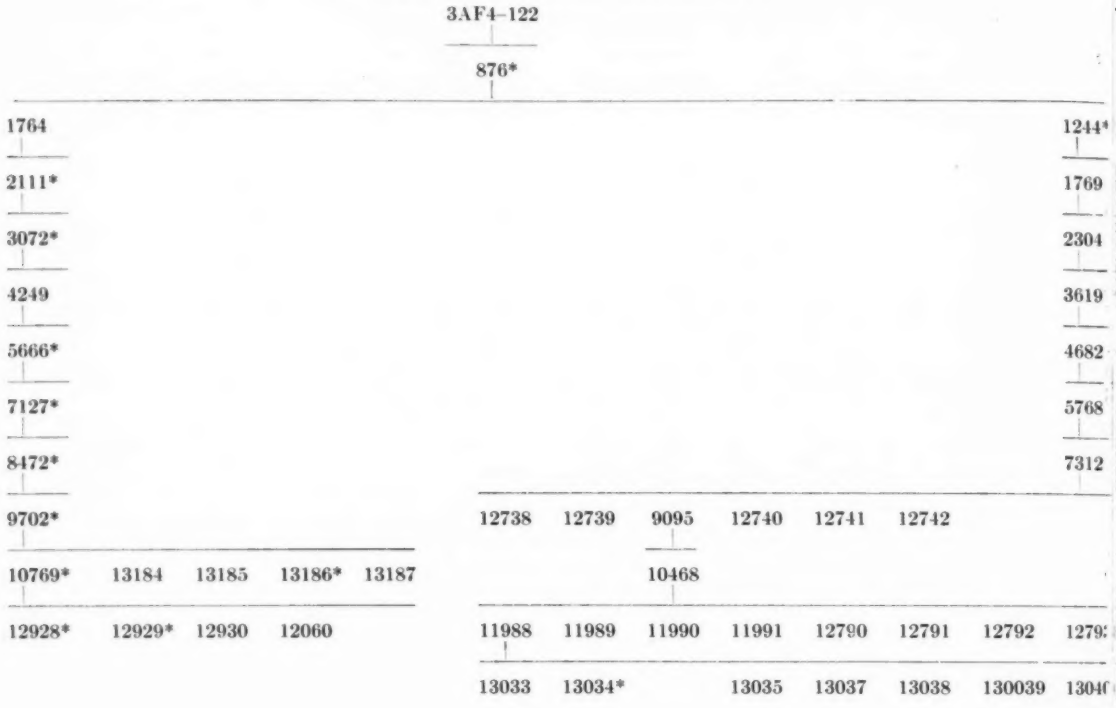
In the establishment of other experiments involving the Marsh strain, females from the 7 families of the first expansion of the stock were used indiscriminately. Warner et al. (*4*), for example, used 26 strain 3 females to produce the hybrids [3] used in a study of mammary-tumor concentration. This group of 26 females was made up of 10 groups of sisters from the various families of the first expansion. Table 3 lists the nonbreeding daughters of these 26 strain 3 mothers and the percentages of tumors observed among them.

When the young of these mothers are compared, it becomes apparent that the young produced by the first three groups of siblings may be characterized as high in tumor production, the next four groups as intermediate, and the last three as low in tumor production.

Table 3 shows that one of the high-cancer and two of the low-cancer groups are members of family A. The high-cancer group traces back

[3] In the following discussion, possible genetic influence due to hybridity is not considered. Hybrids (Marsh albino female × A albino male) are known to be receptive to mammary-tumor inciter (*4*).

PEDIGREE CHART 2.—Family A, showing high- and low-tumor lines of descent in the same family. The animals th developed mammary carcinoma are starred (*).



to female 1482 (a sister of female 1764, pedigree chart 2) in the high-cancer branch of family A, and the two low-cancer groups to females 11988 and 11989 in the low-cancer side of the family.

The three sisters which gave rise to the third low-cancer group (table 3) were descendants of family F. They came from a high-cancer strain of the family. Their mother (female 10760) developed mammary cancer at 625 days, and a sister of their mother, which was not used in hybrid production, developed tumor at 343 days. Possibly these three mice were segregates, although no supporting evidence is available.

The low-cancer groups produced by females of family A were 16 to 17 percent cancerous, and the low group of family F was 11 percent cancerous. These findings, plus the absence of tumors in the descendants of female 12736, give rise to the conjecture that these variations in tumor production may be the result of graded abilities of the mice involved to propagate the mammary-tumor inciter in quantities sufficient to stimulate the mammary tissue to the threshold of cancer.

A study of the appearance of mammary tumors in succeeding generations of the first expansion of the Marsh stock gives the impression of great irregularity. The tumors seem to appear in cycles of varying frequency and intensity. In family D, for example, two generations of cancer are followed by two generations of noncancer, three generations of cancer, and four generations of noncancer. Strain 1, family F, shows an almost unbroken line of noncancer. Strain 1, family A, gives an almost unbroken line of cancer.

This behavior is difficult to explain on the basis of genetic mutation. It happens too frequently and reverts too regularly. It seems more rational to surmise that these differences are the results of varying amounts of extrinsic or intrinsic stimuli acting upon uniform physiologic systems.

EFFECTS OF VARYING STIMULI UPON UNIFORM PHYSIOLOGIC SYSTEMS

Uniform physiologic systems may be produced in two ways: (1) By intensive and prolonged inbreeding; and (2) by the use of first-filial-generation hybrids obtained by crossing two homozygous stocks.

Using the latter technique (Marsh albino female × A albino male), Warner and his co-worker (4)

BLE 3.—*Classification of strains in the first expansion*

Marsh albino mothers [1] Nos.	Virgin daughters [2]	Cancer incidence: Mice with mammary cancer	Cancer incidence: Mice with no mammary cancer	Cancer incidence: Percentage with cancer	Family
HIGH-CANCER GROUP					
	Number	*Number*	*Number*		
1812, 1813, 1814	49	36	13	73.46	A
10883, 10884, 10885	42	30	12	71.43	B
11907, 11908, 11909	30	26	4	86.67	C
INTERMEDIATE-CANCER GROUP					
822, 823, 824	42	23	19	54.76	D
1829	13	6	7	46.15	E
13096, 13099, 13103	9	4	5	44.44	B
13090, 13091	5	2	3	40.00	F
LOW-CANCER GROUP					
3033, 13034, 12136	6	1	5	16.67	A
12137, 12138	18	2	16	11.11	F
11989, 11990, 11991	37	6	31	16.21	A

[1] The groups of Marsh albino siblings in column 1 were the females used by Warner in his Marsh albino × Bittner albino cross (4).
[2] Nonbreeding females obtained from above matings.

demonstrated that the resultant hybrids are receptive to the mammary-tumor inciter.

If similar hybrids (Marsh albino female × A albino male) are stimulated by the inciters transmitted by the A albino, dilute brown, and C57 black stocks, the percentages of tumors listed in table 4 are obtained. These data are interpreted as indicating that these well-known strains transmit mammary-tumor inciters of characteristic effectiveness.

TABLE 4.—*Mammary cancers in first-generation hybrids of Marsh albino mothers when stimulated by mammary-tumor inciters of other stocks*

Hybrid stock	Mammary-tumor inciter stock	Total mice	Cancer incidence: Mice with cancer	Cancer incidence: Mice without cancer	Cancer incidence: Percentage of cancer	Average age of mice at tumor
		Number	*Number*	*Number*		*Days*
Ma × A	Ba	149	138	11	92.60	396
	dba	141	116	25	82.27	558
	Blk	148	7	141	4.73	643

EVIDENCE THAT MARSH ALBINO FEMALES TRANSMIT VARYING CONCENTRATIONS OF MAMMARY-TUMOR INCITER AFTER SEGREGATION HAS OCCURRED

The 26 Marsh albino females used by Warner and his co-workers (4) in producing his Marsh albino × A albino hybrids were taken at random from the first expansion of the Marsh stock. Nine of these trace back to high-tumor lines, 9 to intermediate-tumor lines, and 8 to low-tumor lines (table 3). If the young obtained from these females are classified according to the type of line from which their mothers came, they show the percentage of tumor listed in table 5.

TABLE 5.—*Mammary cancer in first-generation hybrids of Marsh albino mothers when the young were stimulated by mammary-tumor inciters from females in the high, intermediate, and low cancer lines of the Marsh strain*

Hybrid stock	Mammary-tumor inciter from—	Total mice	Cancer incidence: Mice with cancer	Cancer incidence: Mice without cancer	Cancer incidence: Percentage of cancer	Average age of mice at tumor
		Number	*Number*	*Number*		*Days*
Ma × A	High line	121	92	29	76.03	587
	Intermediate line	69	35	34	50.72	642
	Low line	61	9	52	14.75	568

The descendants of these three groups of Marsh females developed tumor in percentages strikingly similar to those of the group to which their mothers belonged (tables 3 and 5). They paralleled in this respect the behavior of the three unrelated stocks (table 4), where variation of inciter potency produced similar effects.

That the variation noted in table 5 was due to the effectiveness of the inciter transmitted and was not caused by variation in the hybrids produced by mothers of the three classifications is indicated by the results obtained (table 6). In this latter distribution, only the daughters of the low-cancer group of table 4 are used. The similarity of results in tables 4 and 6 indicates that all the hybrid daughters of Marsh strain mothers (table 4) regardless of the classification in which they belong, were equally susceptible to the tumor inciter.

TABLE 6.—*Cancer in first-generation hybrids of low-strain Marsh albino mothers when stimulated by mammary-tumor inciters of other stocks*

Hybrid stock	Mammary-tumor inciter from—	Total mice	Cancer incidence: Mice with cancer	Cancer incidence: Mice without cancer	Cancer incidence: Percentage of cancer	Average age of mice at tumor
		Number	*Number*	*Number*		*Days*
Ma × A	Ba	35	30	5	85.71	364
	dba	27	22	5	81.48	551
	Ma	43	7	36	16.28	572
	Blk	28	0	28	0	

It is therefore concluded that the differences in tumor incidence found here are manifestations of variations in the effectiveness of the inciters transmitted by the animals within these classifications.

These changes in inciter potency are apparently effected during the interim between the ingestion of the mother's milk and the time when the host female would ordinarily develop tumor. The amount of change ranges from very small to total and once established tends to remain constant. There seems to be a positive correlation between the effectiveness of the stimulus transmitted and the percentage of tumor produced, with occasional sharp changes in the direction of no tumor. No changes in the opposite, or more tumor, direction have been noted.

It is concluded that the decrease in tumor production in this stock is due to the cumulative effect of such sharp reductions in inciter effectiveness. The underlying cause of these changes does not appear to be genetic. Whether or not these mice are building up an immunity to the mammary-tumor inciter or whether the stock is subject to a hormonal imbalance requires further investigation.

DISCUSSION

One of the premises upon which genetic experimentation in the field of malignancy has been based is the supposition that inbred mouse strains which have reached theoretical homozygosity remain constant in the crude percentage of tumors that they produce under uniform systems of care and breeding.

The Marsh strain at the beginning of the experiments recorded here had reached theoretical homozygosity. Therefore, in the purely genetic sense any change in percentage of tumors found in later generations would be attributed to mutation in one or more loci of their chromosomes.

In the light of our present knowledge of the mammary-tumor inciter, which has been demonstrated to be largely instrumental in determining the percentage of mamary tumor obtained in inbred strains, it may also be reasoned that the changes listed may be attributed to variations in the potency of the inciter transmitted by the females of the Marsh stock.

Carrying this argument further, we may reason that variations in the potency of the mamary-tumor inciter may be attributed to genetic changes which produce physiologies that are not receptive to the inciter obtained through the mother's milk or physiologies that do not propagate or are not capable of propagating the stimulus received to the point where it rises above the threshold of mammary-tumor production.

The behavior of the descendants of female 10960 in the second expansion of the Marsh stock seems most reasonably explained on the last theory. It seems improbable that a mother nursing four daughters indiscriminately would transmit to one of them an inciter less potent than that transmitted to the other three. It is reasoned, therefore, that mouse 12736 received the same amount of stimulus as did her sisters but was unable to nurture it to the point where it stimulated her mammary tissue to become malignant. Whether this inability to propagate the inciter was due to genetic mutation, physiologic unbalance due to a hormonal upset, or to the presence of an inciter deterrent is problematical.

In this regard, the findings in the first expansion, in which varying degrees of the inability to nurture the inciter is indicated, tend to eliminate genetic mutation as the basic cause of these changes. The behavior of the members of the families in the first expansion of the stock when stimulated by inciters of graded potencies from other stocks and the correlation between these results and the percentages of tumor obtained when graded inciters from within the Marsh strain are used are most reasonably explained on the basis of a change in the mammary-tumor inciter rather than on the basis of a genetic mutation affecting the physiology of the host.

This change in inciter potency is apparently effected during the interim between the ingestion of the mother's milk and the time when the host female would ordinarily develop tumor. There seems to be a positive correlation between the effectiveness of the stimulus and the percentage of tumor produced, with occasional sharp changes in the direction of no tumor.

It is therefore concluded that the decrease in tumor production in this stock is due to the cumulative effect of such sharp reductions in inciter effectiveness.

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TRYPANOSOMA CRUZI IN THE TREATMENT OF MOUSE TUMORS[1,2]

By THEODORE HAUSCHKA, LEROY H. SAXE, JR., and MARGARET BLAIR, with the technical assistance of MRS. N. MAXWELL TOWER and MRS. JEAN PALMQUIST, *Institute for Cancer Research, Lankenau Hospital, Philadelphia*

INTRODUCTION

Positive therapeutic results are claimed by Roskin and his collaborators (*1–6*), who since 1935 have employed *Trypanosoma cruzi* endotoxins in the treatment of various malignant tumors in experimental animals and recently also in human patients (*7*). Reports on the successful clinical application of Klyueva's "cancerolytic" *T. cruzi* extract KR6 ((*8*) and personal communications) in the majority of about 60 cancer patients have given impetus to related studies in our laboratories. These experiments, which were started in March 1945, have shown retardation of growth, but no regression of 3 types of transplantable neoplasms in mice infected with *T. cruzi*. Injection of heat-killed trypanosome cultures did not retard tumor growth significantly.

Our experiments represent part of a joint institutional program devoted to chemotherapy of cancer and participated in by the National Cancer Institute, the Lankenau Hospital Institute for Cancer Research, and the Zoology Laboratory of the National Institute of Health.[3]

Summaries of Roskin's earlier work (*6*, *7*) as well as a survey article evaluating the relationship between protozoology and oncology (*9*) are available. Only a condensed recapitulation of the Russian work is needed in the present paper. Starting from the premise that the intracellular leishmanian multiplication phase of *T. cruzi* (Chagas' disease) might cytolyze cancer cells and inhibit tumor growth, Roskin infected mice with trypanosomes and noted greatly reduced growth often followed by complete regression of Ehrlich carcinoma implanted in these mice. Infected mice bearing tumors lived longer than did infected mice without tumors or noninfected tumor controls. The last group all died of cancer, while the first two groups eventually succumbed to Chagas' disease.

To determine whether tumor inhibition by *T. cruzi* was a specific response or only the consequence of a "simple febrile process playing the role of a non-specific therapeutic agent," Roskin inoculated tumor-bearing mice with *Spirochaeta duttoni* (relapsing fever). This infection produced no significant effects, nor was the growth of implanted kidney tumors in guinea pigs retarded by chronic infections of *T. equiperdum*. A bacterial endotoxin, Pyrifer, from the Rosenberg laboratory in Freiburg (*6*) was likewise ineffectual.

Histologic study of Ehrlich carcinoma in mice infected with Chagas' disease revealed large necrotic areas which did not stain differentially, intermingled with scattered groups of cancer cells. Most of the latter contained leishmania forms of *T. cruzi*. Roskin concluded that *T. cruzi* is "organotropic with regard to mouse tumors."

Despite this pronounced effect on malignant tissue, active Chagas' disease with its usual involvement of heart, brain, and other vital organs was of no therapeutic value. Roskin and his associates, therefore, turned their attention to *T. cruzi* endotoxin, derived at first from heat-killed trypanosomes in the serum of infected mice and guinea pigs and later from killed cultures. Freshly prepared endotoxin from all these sources caused a high percentage of complete regressions of implanted Ehrlich carcinoma in mice and implanted Flexner-Jobling carcinoma in rats. Experimental animals were injected intraperitoneally on 4 to 8 consecutive days. The transplantable Krichevski-Sinelnikov sarcoma in rats did not respond to endotoxin.

As in the case of living *T. cruzi* infections, the histologic changes in the endotoxin-treated tumors were pronounced. Three days after treatment there was lymphocytic infiltration around the growing tumor. Scattered areas of necrotic cells increased in extent during the next few days. At 10 days the tumor was largely replaced by infiltration of fibroblasts and granulation tissue.

Since tumor fragments soaked in endotoxin for 6 hours prior to implantation failed to take, while control pieces soaked in serum grew regularly, Roskin assumed direct destruction of cancerous tissues by his preparation. Treatment of tumorous mice previously splenectomized or injected with trypan blue was ineffectual and suggested to Roskin an indirect stimulatory action also of *T. cruzi* endotoxin on the reticulo-endothelial system.

Gradual refinement of the original crude endotoxins was largely the work of Klyueva (*8*). The improved preparations, especially KR6, have been tested clinically in about 60 cancer patients in Russia ((*7*) and unpublished case reports). Repetition and extension of Roskin's work, therefore, appears amply justified. Our own experiments are aimed primarily at determining the specificity limits and variations in the effectiveness of *T. cruzi* infections and endotoxin with regard to trypanosome strain, type of tumor, and susceptibility of host.

[1] Received for publication November 11, 1946.
[2] Aided by a grant from the National Cancer Institute.
[3] Dr. T. B. Dunn, of the National Cancer Institute, provided us with uniformly behaving transplantable mouse tumors. Miss Eleanor M. Johnson, Zoology Laboratory, National Institute of Health, performed exploratory experiments on virulence, selected cultures of *T. cruzi*, and familiarized us with improved culture procedures that she had recently developed. Dr. Murray J. Shear, of the National Cancer Institute, began and organized this cooperative project.

MATERIALS AND METHODS

Trypanosoma cruzi

A culture of *Trypanosoma cruzi* ("Brazil strain"), originally isolated from a human patient in Brazil, was obtained from the National Institute of Health in December 1945. Preliminary tests by Miss Eleanor Johnson had shown this strain to be lethal to C3H mice of various age groups. The trypanosomes were maintained in bacteria-free culture at room temperature in her diphasic modification of Senekjie's medium (*10*), which consists of a solid agar base containing Bacto-beef (Difco), Neopeptone (Difco), and defibrinated rabbit blood and a liquid overlay of Locke's solution. The pH was adjusted to 7.2–7.4. Maximum growth in this medium was attained in 12 to 18 days. Subcultures were regularly made during the peak of the logarithmic growth phase. Through 39 consecutive transfers the culture characteristics of the strain remained unchanged. No change in virulence became evident during this period.

Mice were infected by intraperitoneal injection of 0.2–0.3 cc. overlay from 2-week-old cultures. At this time, the population counts, as determined by hemacytometer, averaged 82,000 organisms, mostly crithidial, per cubic millimeter, i. e., from 16 to 25 million organisms per infectious inoculum.

The Brazil strain of *T. cruzi* grew less abundantly in Roskin's liquid medium which consists of citrated human blood and Ringer-Locke solution (*6*). Infections of about equal intensity resulted from inoculating mice with trypanosomes grown in either of the foregoing two media.

Trypanosoma cruzi "Endotoxin"

Endotoxin was prepared from uncontaminated cultures grown in modified Senekjie's medium in 250-cc. and 2,000-cc. Erlenmeyer pyrex flasks. The densely populated overlay was withdrawn from 2-week-old cultures and was stored in test tubes overnight at 6°–7° C. The tubes were then immersed for 30 minutes in a water bath maintained between 48° and 51°. No trypanosomes survived this heat treatment. The suspension of killed organisms in culture fluid contained the endotoxin (*6*). The temperature range from 43° to 47°, tested in preliminary experiments, was found unsafe, for some organisms survived and were able to produce infections.

Endotoxin was never more than 24 hours old when administered and was injected intraperitoneally on four consecutive days as follows: First day, 0.3 cc.; second day, 0.3 cc.; third day, 0.4 cc.; fourth day, 0.4 cc. per mouse. This corresponds to the effective dosage reported by Roskin (*6*). In one experimental series, each mouse received 7 cc. of endotoxin per week for three consecutive weeks.

Tumors and Mice

Three kinds of transplantable tumors and two varieties of spontaneous neoplasm served as test objects.

The squamous epithelial carcinoma 119 grows only in strain A mice. When we obtained this tumor from Dr. Dunn, it had been transplanted through 64 generations. Since then it has been transplanted regularly every 2 weeks and is now in its eighty-seventh generation (November 1946). Carcinoma 119 seldom fails to take, grows at a relatively slow rate, and may ulcerate when still small. The regularly cystic center contains cellular debris and leukocytes. In tumors infected with *T. cruzi* such necrotic regions could, therefore, not be interpreted as tissue destruction caused by the parasites.

A C3H mammary adenocarcinoma, established as a transplantable tumor by Dr. Dunn, was received by us after two transfers and is now in its eighth generation (November 1946). This neoplasm grew uniformly, but more slowly than carcinoma 119. Stock tumor is being maintained in C3H female mice by transplantation every 4 weeks.

Sarcoma 37 is less specific in its host requirements and grows faster than the foregoing two neoplasms. Having originated from a mammary adenocarcinoma in which the connective-tissue stroma elements were transformed into sarcoma cells (*11*), this tumor has survived since 1911 in mice of various genetic backgrounds. Several mice bearing sarcoma 37 were supplied by Dr. I. C. Diller. The tumor is being transplanted every 7 days.

Spontaneous adenocarcinoma of the breast occurs in a high percentage of female C3H and dba mice after they have passed the age of 6 months. For our experiments, 140 mice with tumors of comparable size and site were selected from a colony of over 1,000 C3H females. Only 11 dba mice with breast tumors were treated.

A total of 696 experimental mice was divided as follows: 146 male and female A mice, 1 to 3 months old (carcinoma 119); 189 female C3H mice from brother-sister matings, 2 to 4 months old (transplantable adenocarcinoma of the breast); 140 female C3H mice and 11 female dba mice, 6 to 10 months old (spontaneous mammary tumors); 45 female white mice of unknown genetic background 2 months old (sarcoma 37); 165 female white mice similar to the preceding lot (toxicity tests).

EXPERIMENTAL PROCEDURE

In each experimental series, test and control mice were matched with regard to strain, sex, age, and weight. Whenever possible, litter mates were evenly distributed. Test and control transplants were always derived from the same individual

tumor. Tumor pieces, removed by sterile technique and cut about 2 mm. in diameter, were transplanted subcutaneously by trocar in the flank about halfway between the right fore and hind limbs.

Test mice were infected by intraperitoneal inoculation from 2-week-old *T. cruzi* cultures. The endotoxin preparations were also injected intraperitoneally. Dosages are referred to previously.

The long and short diameters and depth of tumors were measured with vernier calipers at regular intervals. Approximate tumor volumes were calculated from these measurements.

Thick blood smears from the tail tips of all infected and of some endotoxin-treated mice were made every 10 days. These smears were air-dried for several hours, then laked in neutral distilled water, and stained with Giemsa. Trypanosome counts were based on examination under oil of 100 fields per slide. In each series, a few mice were killed at different stages of the experiment to obtain tissues from tumors and various organs for histologic study. Particular attention was paid to spleen weights. Tissues were fixed in Zenker's fluid, paraffin-sectioned 6μ thick, and stained with Mayer's hemalum and eosin.

Correlation was sought between tumor growth, course of infection, and changes in body weight.

RESULTS

Course of Infection

Strain, age, and sex of mice, as well as amount of infectious inoculum, are among the principal factors determining the course of infection. Under our experimental conditions, *Trypanosoma cruzi* (Brazil strain) was more virulent for A mice than for C3H mice. When injected with about 20 million trypanosomes per individual, most A mice died in from 30 to 50 days, while C3H mice usually survived for the duration of the experiments (90–120 days).

Weanling A mice began to die off rapidly 10 to 14 days after infection, while 8-week-old animals of the same strain generally lived beyond 3 weeks. Judged from the number of trypanosomes in the blood and leishmanian stages in the heart muscle, the disease was about equally severe in C3H mice ranging from 8 weeks to over a year in age and was usually not lethal within 3 to 4 months. At the end of this period, the survivors were liquidated.

Trypanosome counts based on thick smears from the peripheral blood of 95 infected mice (100 fields under oil per slide, or number of trypanosomes per 1,000 leukocytes) indicated a rather uniform course of the infection, as follows: Trypanosomes (fig. 1) appeared in the blood 7–10 days after intraperitoneal injection of culture fluid. Slender forms (fig. 2) were relatively frequent at this stage of the disease. The blood population increased

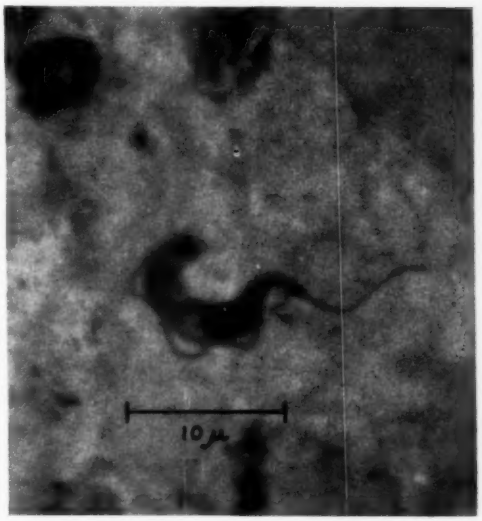

FIGURE 1.—*Trypanosoma cruzi*, stained with Giemsa, against background of laked red cells.

until a peak was reached between 25 and 35 days. This was followed by a decrease. At 88 days, 11 of 19 infected C3H female mice failed to reveal parasites in thick blood smears. Two months earlier, the blood of all these animals had been heavily infected.

As may be expected, the number of trypanosomes in the blood varied directly with the infectious dosage. Twenty days after intraperitoneal injection of 0.1 cc. of culture, the blood count averaged 6 trypanosomes per 100 fields under oil (average based on 16 mice). When the injection was 0.3, 0.5, and 1.0 cc., respectively, the corresponding twentieth-day counts were 32 (average based on 30), 48 (average based on 10), and 159 (average based on 20) trypanosomes per 100 fields. The largest individual count recorded was 616 per 100

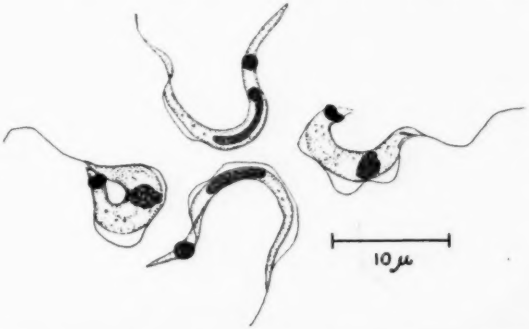

FIGURE 2.—Broad and slender forms of *Trypanosoma cruzi*, drawn with camera lucida from thick smear stained with Giemsa.

fields in a 13-month-old C3H male, which had been injected with 1.0 cc. of culture 30 days earlier. The blood-population levels attained by *T. cruzi* in mice fall far short of the enormous density characteristic for other trypanosomes, such as *T. equiperdum*.

Compared with healthy controls, animals with Chagas' disease appeared somewhat listless, especially during the third and fourth week of infection. Infected A mice often suffered from diarrhea. Mice that survived beyond 3 weeks lost weight during and after the peak of infection. Later much of the lost weight was regained, perhaps as a result of acquired partial immunity. Twenty C3H female mice, which had been infected for 46 days, averaged 7 gm. less in body weight than did 20 controls of similar initial weight. The spleens of infected animals were consistently larger than normal spleens. Spleens of 15 infected C3H female mice averaged 277 mg. (range 148–400 mg.); the average for 15 controls was 224 mg. (range 118–305 mg.), a difference of 24 percent. Heart muscle, taken during the height of the disease, always contained pockets of leishmanian and crithidial stages of *T. cruzi*. Parasites were occasionally found in the circular muscle layer of the small intestine adjacent to the submucosa and in the collagenous connective tissue composing the bronchiolar walls of the lung. Liver, spleen, brain, kidneys, and ovaries were not found parasitized. The histology of infected tumor tissue is briefly described in the next section.

Effect of Trypanosoma Cruzi Infection on Tumor Growth

Carcinoma 119 in A Mice

Sixty A mice 2 months old were evenly divided according to sex and litter into 3 groups: Group 1, the test group, consisting of 10 males and 10 females, was implanted with squamous epithelial carcinoma 119 and infected 2 days later with 0.2 cc. of *T. cruzi* culture; group 2, a tumor-control group of 10 males and 10 females, was implanted with pieces from the same individual tumor as the test; group 3, a third group of 10 males and 10 females, served as *T. cruzi* control, having been injected with the same culture and at the same time as the test group. Body weights of mice were not recorded for this experiment. One male and one female from each group (6 mice) were killed for histologic study.

All mice in groups 1 and 3 became infected, and the counts for *T. cruzi* in the blood were relatively uniform. The tumors in the infected mice were consistently smaller than in the controls. Figure 3 depicts the characteristic difference in tumor growth 3 weeks after the beginning of the experiment. Both test and control tumors grew somewhat faster in female than in male hosts (fig. 4).

Figure 3.—Inhibition of carcinoma 119 in A mice by Chagas' disease. Control tumor (left) is considerably larger, while the test tumor shows less growth. Photographed on twenty-first day of experiment.

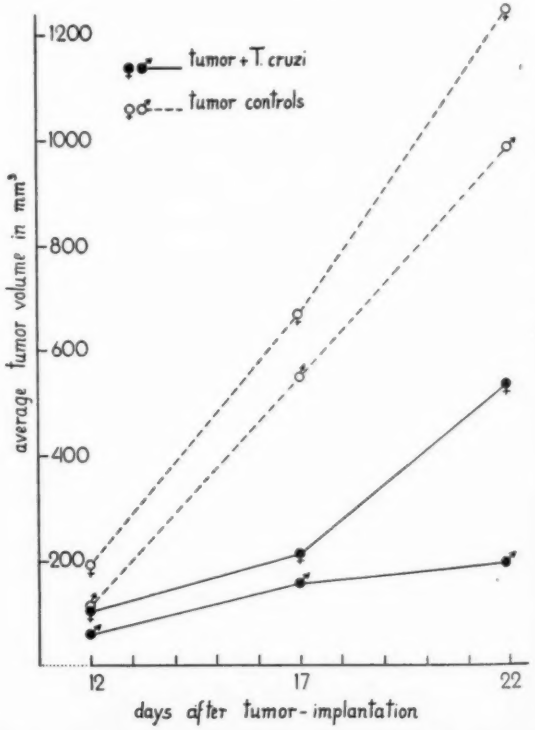

Figure 4.—Growth of carcinoma 119 in strain A mice, as affected by *Trypanosoma cruzi* and by sex of litter-mate hosts (based on 40 mice).

At 17 days, control tumors were about 200 percent larger than the test tumors.

Despite their smaller tumors, the test animals died before the controls (fig. 5). In all 3 groups the females outlived the males. After 61 days all males were dead, while 12 of 27 females were still living.

The foregoing experiment was repeated with similar results in a group of eight strain A litter mates 1 month old in order to obtain comparable tissues for histologic study. Hearts as well as tumors of the infected mice were parasitized. In

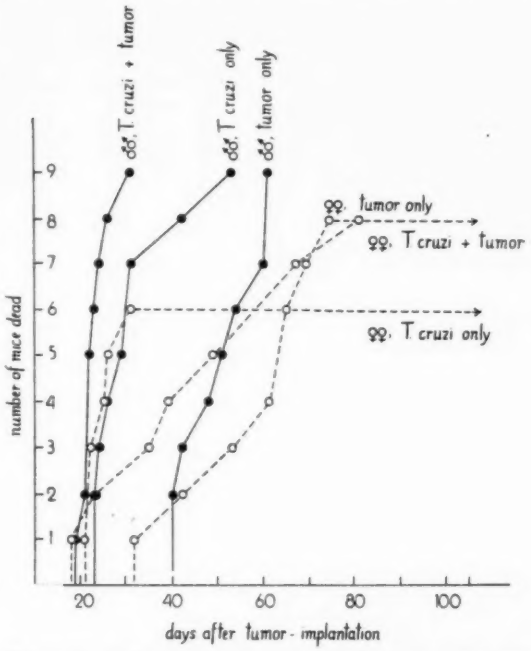

FIGURE 5.—Mortality in A mice implanted with squamous epithelial carcinoma 119 and/or infected with *Trypanosoma cruzi* (based on 54 mice).

the tumors Leishmania forms were found mainly in the stroma and in the encapsulating connective tissue. Infected cancer cells (figs. 6 and 7) were rare and were found only in the peripheral region of the tumor. The central portions of both test and control tumors were characteristically cystic and contained much cellular debris and leukocytes. There was no indication of necrosis caused by parasitism.

MAMMARY ADENOCARCINOMA IN C3H MICE

Thirty C3H female mice 7 to 8 weeks old and 30 female mice 18 weeks old were divided into 3 groups as follows: Ten older and ten younger females were implanted with tumor and inocu-

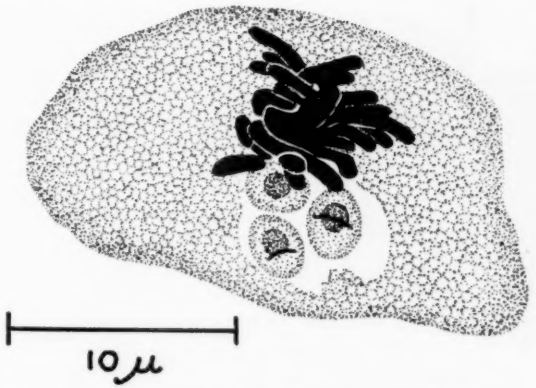

FIGURE 6.—Leishmania forms of *Trypanosoma cruzi* in a cancer cell (carcinoma 119). Chromosomes of host cell are in metaphase. Drawn with camera lucida from tumor section stained with Mayer's hemalum and eosin.

lated with 0.2 cc. of *T. cruzi* culture 9 days later. Ten females of each age group were implanted with tumor only, and 10 females of each age group were given the infection only. Fairly uniform trypanosome counts were obtained from the blood of all infected animals. Tumor growth in the test group was again consistently retarded, as is evident from table 1 and figure 8. There was no difference in survival between the 20 infected and the 20 noninfected tumor-bearing mice (fig. 9), but among the 20 *T. cruzi* controls there was only 1 death. The *T. cruzi* control series was discontinued after 90 days of infection and implanted with mammary tumors to evaluate the effect of an old infection.

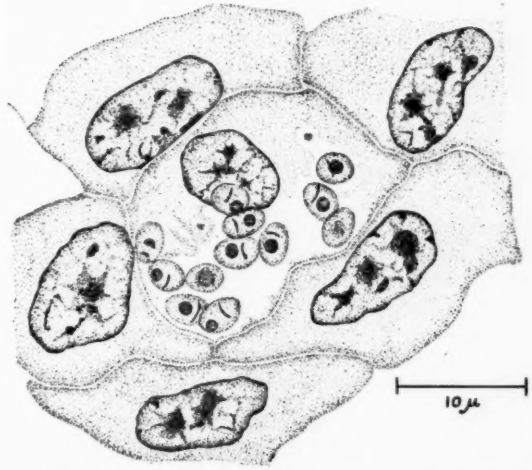

FIGURE 7.—Tumor cell containing 12 parasites. Drawn with camera lucida from section through carcinoma 119, stained with Mayer's hemalum and eosin.

FIGURE 8.—Inhibition of transplantable mammary adenocarcinoma in C3H mice with *Trypanosoma cruzi*. Infected mouse on right. Photographed on forty-fifth day of experiment.

After 3 months of infection, the surviving 19 *T. cruzi* controls were implanted with mammary adenocarcinoma, as were 20 noninfected C3H females of comparable age. Blood smears and tumor measurements were taken at 10-day intervals up to 60 days after tumor transplantation. Although the infection was of long standing in this experiment and less than half of the blood smears were positive for trypanosomes, the tumors were consistently smaller in the infected mice. At 50 days, the test tumors averaged 918 mm.^{3}, as compared with 1,228 mm.^{3} in the controls. The old infection did not retard tumor growth as markedly as did Chagas' disease introduced a few days after tumor implantation.

Tumors grew faster in the youngest C3H hosts. This faster growth held true for both tests and controls (fig. 10). Tumors in all three control

TABLE 1.—*Volume of transplanted adenocarcinoma of the breast in female C3H mice as affected by* Trypanosoma cruzi

Tumor volume at —					
21 days		31 days		41 days	
Test (in cubic millimeters)	Control	Test	Control	Test	Control
	Cubic millimeters	*Cubic millimeters*	*Cubic millimeters*	*Cubic millimeters*	*Cubic millimeters*
310	252	576	1,438	1,912	3,080
300	270	422	796	1,240	1,896
280	130	340	722	684	1,286
80	98	252	596	632	1,264
56	96	184	336	510	1,110
30	86	98	328	344	1,040
20	52	84	232	102	804
[1]	32	58	82	78	552
[1]	[1]	[2]	[1]	26	186
[1]	[1]	[1]	[1]	[2]	[2]
Average 154	*Average* 124	*Average* 252	*Average* 554	*Average* 614	*Average* 1,256

[1] Tumor not palpable. [2] Tumor too small to measure.

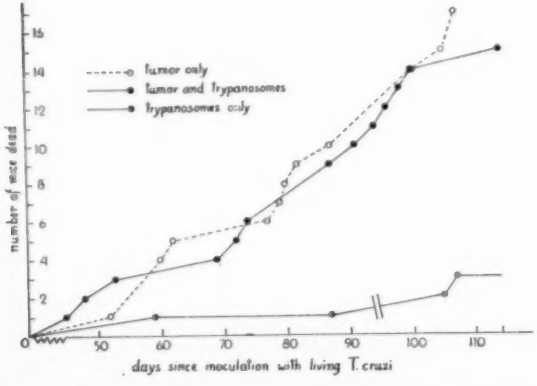

FIGURE 9.—Survival of C3H mice implanted with mammary carcinoma and infected with *Trypanosoma cruzi* (based on 60 mice).

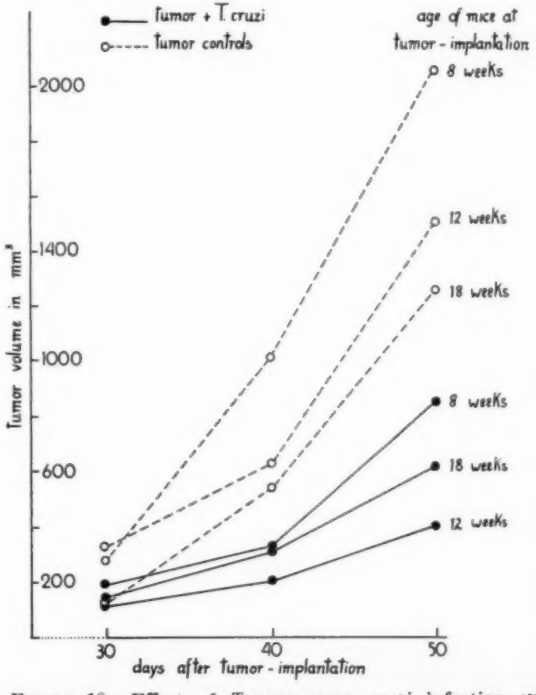

FIGURE 10.—Effect of *Trypanosoma cruzi* infection on growth of mammary carcinoma in C3H female mice (based on 60 mice).

groups of different age were two to three times as large as in the corresponding test groups.

No parasites were found in any of the experimental mammary neoplasms, while heart and other vital organs, such as lungs and intestine, showed multiplication stages of *T. cruzi*. The smaller volume of the test tumors coincided with a rather drastic loss of body weight during the height of the infection (fig. 11). There were 10

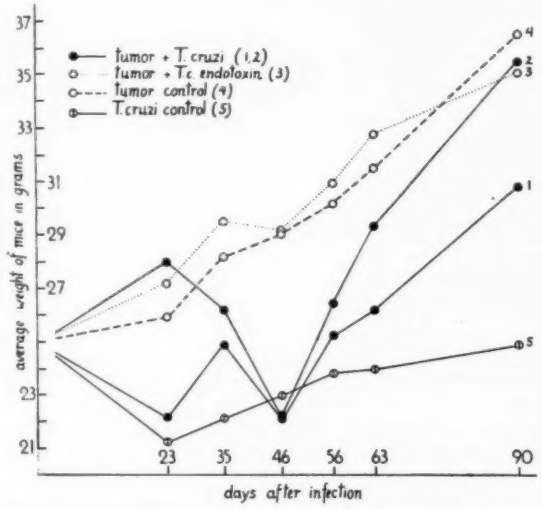

FIGURE 11.—Weight changes in tumor-bearing or infected C3H female mice (based on 45 mice).

mice each in groups 1–4, and 5 in group 5. Groups 1 and 5 were heavily infected with *T. cruzi*, whereas group 2 had a relatively light infection. All mice averaged about 25 gm. in weight at the beginning of the experiment.

SARCOMA 37

Forty-five female white mice of unknown genetic constitution, 2 months old, were equally divided into 3 groups: Group 1 was implanted with sarcoma 37 and infected with 0.3 cc. of *T. cruzi* culture; group 2 received the tumor only, and group 3 the infection only. At 10 days the blood count for the infected groups averaged 9 trypanosomes per 100 fields under oil. No weight loss was evident at 5, 10, and 15 days. Tumor growth in the test group was consistently retarded. On the fifteenth day, test tumors averaged 266 mm.^{3}, as compared with 744 mm.^{3} in the controls, a difference of almost 200 percent. This experiment is still in progress.[4]

[4] Sections through tumor and heart obtained on the seventeenth day of this experiment showed no parasites. Since the mice had not lost weight and the trypanosomes in the blood were relatively few (32 per 100 fields), inhibited growth of sarcoma 37 appears all the more significant.

SPONTANEOUS MAMMARY TUMORS

In a small group of 15 C3H female mice with spontaneous mammary adenocarcinoma of comparable size and site, *T. cruzi* infection slowed tumor growth only slightly.

EFFECT OF TRYPANOSOMA CRUZI "ENDOTOXIN"

Endotoxin was prepared from *T. cruzi* cultures (Brazil strain, grown in modified Senekjie's medium) according to the method outlined previously. The preparation was not noticeably toxic to mice of various strains, and no anaphylactic phenomena were observed in animals previously sensitized by repeated injections of heat-killed *T. cruzi* cultures. Intraperitoneal injections of 1.0 and 2.0 cc. (equivalent to 50–160 million trypanosomes per inoculum) spaced over 3 weeks, until a total of 21.0 cc. had been administered, were tolerated by most mice. The number of deaths in a series of 125 C3H females and 15 A females so injected was no greater than the mortality in control groups treated with equal amounts of culture fluid without trypanosomes. (Roskin likewise reported no toxic effects of his endotoxin preparations and made no mention of demonstrable antibodies to *T. cruzi* materials.)

Soaking of tumor pieces in *T. cruzi* endotoxin for 6–8 hours at room temperature prior to implantation did not interfere with subsequent tumor growth. Pieces soaked in endotoxin took and grew as well as did controls immersed in Locke solution containing diffusible components of sterile blood-agar culture base. This was established for both carcinoma 119 (in 34 A mice) and mammary adenocarcinoma (in 20 C3H mice).

Forty A mice 6 weeks old were equally divided according to sex and litter and were implanted with carcinoma 119. The 20 test mice received 4 intraperitoneal injections of freshly prepared endotoxin (0.3, 0.3, 0.4, and 0.5 cc.) on 4 consecutive days following tumor transplantation. The 20 controls were injected with appropriate control fluid. This corresponds to the effective dosage reported by Roskin (*6*). There was no significant difference between tumor growth in the 2 groups, except that the endotoxin-treated tumors ulcerated somewhat sooner. Earlier ulceration accounts for the fact that after 11 weeks, 14 of 20 test mice had died, as compared with 8 of 20 controls.

To compare the effect of the living *T. cruzi* infection with the influence of endotoxin, 45 C3H female mice 4 to 6 months old were divided into 5 groups as follows: Group 1, 10 mice having a relatively heavy infection and implanted with mammary adenocarcinoma; group 2, 10 mice with a relatively light infection and tumor; group 3, 10 mice with tumor, treated with 4 consecutive injections of 0.3, 0.3, 0.4, and 0.5 cc. of endotoxin;

group 4, 10 mice with tumor only; and group 5, 5 mice with *T. cruzi* only.

Groups 1 and 2 had been "sensitized" with four injections of heat-killed *T. cruzi* cultures spaced over a period of 6 weeks, before they were inoculated with living trypanosomes. This preliminary treatment did not immunize against Chagas' disease. As in earlier experiments, the tumors in groups 1 and 2 were consistently smaller than in the control series 4. Weight changes for this experiment are plotted in figure 11. Tumor increment in the endotoxin series 3 (fig. 12) coincides very closely with the growth curve for untreated controls.

One hundred twenty-five C3H females and eleven dba females with spontaneous breast tumors of comparable size and site were divided into 2 groups of 68 mice. The test group was treated with massive dosage of endotoxin, i. e., a total of 21.0 cc. spaced over 3 weeks. About two-thirds of the controls were injected with equivalent amounts of Locke overlay, and the rest remained untreated. During the 3 weeks of treatment, 19 test and 21 control mice died. Tumor growth in the remaining 49 test and 47 control mice showed similar trends, regardless of treatment (fig. 13). During

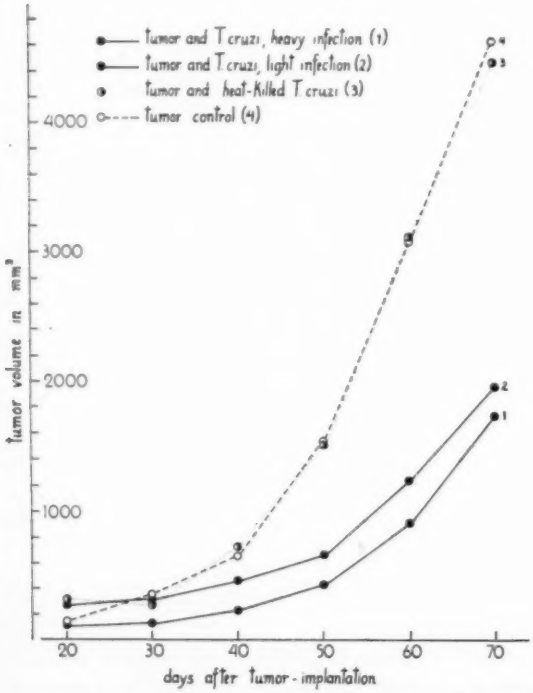

FIGURE 12.—Growth of adenocarcinoma of breast in C3H female mice, as influenced by *Trypanosoma cruzi* and *T. cruzi* endotoxin (based on same 40 mice, the weights of which are plotted in figure 11, groups 1–4).

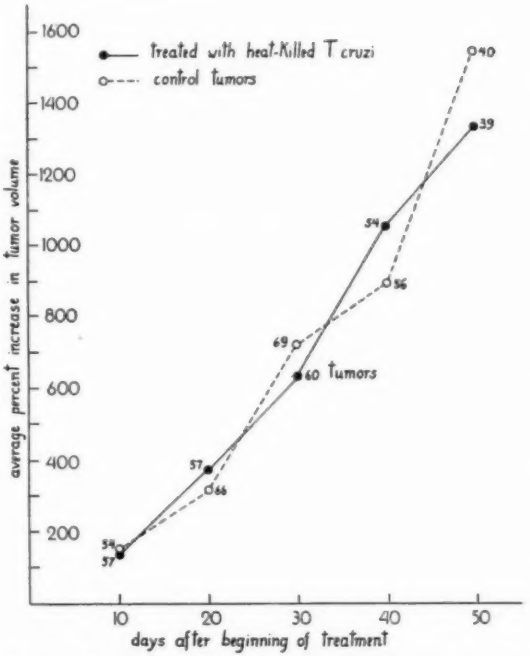

FIGURE 13.—Growth of spontaneous adenocarcinoma of breast in C3H and dba mice, unaffected by treatment with *Trypanosoma cruzi* (Brazil strain) endotoxin (based on 96 mice with more than 100 spontaneous tumors). Number of tumors measured is indicated for each point on the chart.

the 10 days following the 21-day treatment period, there were 12 deaths in the test group and only 3 deaths among the controls. This difference was compensated for by slightly higher mortality among the controls from the thirty-first day onward. In addition to the tumors present at the beginning of this experiment, a total of 17 new tumors was recorded in the test animals and 25 in the controls during 50 days of observation.

DISCUSSION

Discrepancies between the results obtained by us with the Brazil strain of *Trypanosoma cruzi* and Roskin's more positive findings (*6, 7*) are not to be considered as contradicting the Russian experiments. Roskin observed regressions in a variety of transplantable and spontaneous neoplasms in mice, rats, and man. It should be stressed here that Roskin's effective *T. cruzi* endotoxins, like our ineffective preparations, produced no toxic symptoms in the treated mice or rats, with the possible exception of a small group of mice in which the reticulo-endothelial system was experimentally blocked. Roskin's endotoxins were effective, whether prepared from the blood of infected mice and guinea pigs or from trypanosomes cul-

tured in citrated human blood. We can therefore rule out tumor variety, host species, toxicity of endotoxin, and culture method as of secondary importance in determining therapeutic activity.

This points toward strain specificity of *T. cruzi* as the possibly decisive factor in endotoxin therapy of malignant tumors. The existence of biochemical mutants varying in nutritional requirements, production of metabolites, or virulence is well-established both for nonpathogenic and pathogenic microorganisms. Different strains of *T. cruzi* range widely in virulence (*12*) and in their affinities for one or more host tissues. Thus, Brumpt (*13, v. 1, p. 341*) ascribes to *T. cruzi* "une affinité toute spéciale pour celles du système réticulo-endothélial." The considerable variability of symptoms in human patients is partly determined by the preferential localization of parasites in certain organs. A neurotropic strain of *T. cruzi*, investigated by Campos (*14*), produced focal inflammatory lesions in the spinal cords of dogs. This strain retained its selectivity for neural tissue through 1 year of continuous culture on blood-agar medium and continued to produce paraplegia in mammals also after experimental transfer through the invertebrate host, *Triatoma megista*. A special affinity for Ehrlich carcinoma is suggested by the "positively tumorotropic" behavior of *T. cruzi* in Roskin's laboratory (*6*). The Brazil strain used in our experiments preferred muscle and connective tissue to squamous epithelium. Cancer cells were parasitized only exceptionally.

The consistent retardation of tumor growth by *T. cruzi* (Brazil strain), is, therefore, not attributable to direct destruction of tumor tissue by parasites but may be ascribed to a variety of nonspecific causes, viz: Nutritional depletion of host system as expressed by weight loss; infection of vital organs, especially the heart, with consequent impairment of circulation; interference with proper vascularization of tumor due to destruction of parasitized connective tissue elements around the tumor which would normally contribute toward the formation of new blood vessels; toxic metabolites (*13 v. 1, p. 354*) produced by the rapidly multiplying trypanosomes; and competition for growth substances required by the tumor cells.

SUMMARY

Infections of *Trypanosoma cruzi* (Brazil strain) significantly retarded the growth of three transplantable tumors, squamous epithelial carcinoma 119 in A mice, mammary adenocarcinoma in C3H mice, and sarcoma 37 in white mice of unknown genetic background. Spontaneous breast adenocarcinoma in C3H mice was lightly retarded in growth. The infection was more virulent in A than in C3H mice.

The inhibitory effect appears to be nonspecific, since it was frequently accompanied by loss in body weight and parasitemia of vital organs such as heart, lungs, and intestine.

Cancer cells were rarely parasitized. Leishmanian and crithidial forms of *T. cruzi* were more abundant in the stroma and the connective tissue surrounding some tumors.

Retardation in tumor development did not result in longer survival.

Endotoxin prepared from heat-killed cultures of *T. cruzi* (Brazil strain) was without effect against carcinoma 119 or against transplantable and spontaneous mammary tumors.

Our findings are not to be considered as contradicting the results of Roskin and Klyueva, inasmuch as their strain of *T. cruzi* has so far not been available to us.

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EFFECT OF DIETS DEFICIENT IN CERTAIN AMINO ACIDS ON THE INDUCTION OF LEUKEMIA IN dba MICE [1] [2]

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There is no doubt that the latent period of the induction of various types of tumors in both rats and mice can be altered by a variety of dietary restrictions. Tannenbaum (*1*) established that the level of caloric intake is an important factor in the formation of tumors in mice. Restriction in caloric intake resulted in a marked decrease in the incidence of every type of tumor studied. These results were substantiated in other studies (*2, 3, 4*). Other investigators (*5, 6*) showed that certain vitamins of the B group can prolong the latent period of tumor formation. We showed (*7, 8, 9*) that the restriction of dietary cystine markedly retards spontaneous mammary tumor formation in strain C3H mice and induced leukemia in the strain dba mice.

The retardation of body growth of young animals fed diets restricted either in calories or cystine does not as such explain why these animals develop fewer tumors and at a later time than do the controls on a complete diet. Young mice maintained on a diet restricted in cystine and in which no body growth was apparent, showed no mammary tumors within a lifetime of 22 months, while the controls showed an incidence of almost 100 percent. Yet when the mice on the restricted diet were treated with stilbestrol, the tumor incidence rose to 44 percent (*10*). This striking difference indicated that the dietary deficiency reduced the tumor incidence by affecting certain glands of internal secretion directly and hence tumor genesis indirectly. Studies indicated that the mice on the restricted diets were in anestrus, that the mammary tissue failed to develop in the virgin and atrophied in the breeding female, and that implantation of stilbestrol pellets in these mice resulted in growth of mammary tissue, continuous estrus, and a rise in tumor incidence (table 1).

TABLE 1.—*Effect of subcutaneously implanted pellets of diethylstilbestrol upon spontaneous mammary tumors in strain C3H virgin female mice* [1]

[Numerators=number of mice developing tumors. Denominators=number of mice dying free of tumors]

Regimen	Effective number of mice	Mice developing tumors or dying free of tumors according to age (in months)															Tumor incidence	Average age of tumors
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	22	Total	
Mice without pellet:																	*Pct.*	*Mos.*
Chow	350	3/0	11/1	20/0	35/0	63/0	51/0	56/1	41/1	26/1	13/1	10/2	5/0	6/0	1/1	0/1	341/9	97.4	10.4
High cystine	42	0/0	11/1	5/0	9/0	6/0	2/0	4/0	3/0	1/0	.....	.....	.....	.....	.....	.....	41/1	97.6	8.3
Low cystine	45	0/0	0/0	0/0	0/0	0/0	0/0	0/3	0/2	0/0	0/1	0/0	0/2	0/1	0/0	0/0	0/9	.0	.....
Mice with pellet:																			
Chow	27	0/0	4/0	3/0	2/0	3/0	4/0	3/0	3/0	1/0	0/1	.....	.....	.....	.....	.....	23/1	95.8	9.1
High cystine	28	0/1	3/1	4/0	7/0	3/0	1/0	5/0	0/0	0/0	0/0	.....	.....	.....	.....	.....	23/2	92.0	8.4
Low cystine	40	0/0	0/4	2/3	2/2	3/1	1/1	1/1	1/1	2/1	3/1	1/1	0/1	1/4	.....	.....	17/21	44.7	11.1

[1] Data taken from White and White (*10*).

Young mice placed either on a cystine-restricted or lysine-restricted diet may be equally retarded in growth. Painting with methylcholanthrene resulted in a low incidence of leukemia in the former group, while the incidence of leukemia in the latter was nearly as high as in the controls. These data indicate that while dietary restriction of cystine results in a reduction of induced leukemia, a lysine-restricted diet has no apparent effect and that the mechanism for these differences may be due to an effect on some hormonal system (table 2).

TABLE 2.—*Effect of diet on the response of dba mice to painting with methylcholanthrene* [1]

Diet	Number of mice	Mice with leukemia			Mice with sclerosis		
		Number	Percent	Mean latent period	Number	Percent	Mean latent period
				Days			*Days*
Dog chow	40	36	90	99	0	.....	.....
High cystine	40	36	90	101	0	.....	.....
Low cystine	40	4	10	127	32	80	144
High lysine	36	28	78	101	2	5.6	.....
Low lysine	36	26	72	104	2	5.3	.....

[1] Data taken from White, Mider, and Heston (*8*).

The marked difference in response by mice on these two regimes suggests that cystine played a

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role in the development of induced leukemia perhaps not associated with its effect as an essential amino acid for growth but with some other undetermined property.

Weissman and Schoenheimer (*11*) have shown that lysine labeled with N^{15} and administered to rats can be steadily deaminated and yield its nitrogen for the formation of other amino acids. This is also true of other essential amino acids studied. However, only in the case of lysine does the reverse of this process not take place, and lysine is never regenerated but must be supplied as such in the diet. Because of this unusual property of lysine, it was felt that the study of the effect of some other essential amino acid on the incidence of induced leukemia was desirable.

Accordingly, three diets were prepared in such a manner that the amount of cystine, lysine, and tryptophane, respectively, would be considerably restricted to allow no growth (increase in weight) but indefinite maintenance. These same diets, each supplemented with the amino acid in which it was restricted, permitted good growth. Another group of mice was fed a Purina dog chow diet.

Table 3 contains the various ingredients used in each diet. Inasmuch as the cystine-restricted diet was somewhat different from the one employed previously (*7*), an additional comparison was made between the new cystine-restricted and the "old" cystine diets. In our previous studies, we had used Ryzamin B No. 1 as part of our source of the vitamin B supplements and liver extract (Lilly 343) to supply the remainder. However, the manufacture of Ryzamin B No. 1 was discontinued and was replaced by a Ryzamin B No. 2, which is a preparation nearly complete in the B vitamins needed. The use of liver extract was therefore discontinued.

TABLE 3.—*Composition of the diets*[1][2]

Diet constituent	Diet group									
	1	1a	2	2a	3	3a	4	4a	5	6
	Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
Labco (casein vitamin-free)	3.9	3.9	5.0	5.0	3.0	3.0	....	....	....	....
Casein, peroxide-treated	....	....	....	....	7.0	7.0	....	....	....	....
Gliadin	....	....	....	....	....	....	18.0	18.0	14.0	....
Corn starch	55.1	54.6	66.0	65.6	60.72	60.62	53.0	52.38	57.0	....
Crisco	22.0	22.0	15.0	15.0	15.00	15.00	15.0	15.0	15.0	....
Cod-liver oil	3.0	3.0	....	....	....	....	....	....	....	....
Corn oil	....	....	5.0	5.0	5.0	5.0	5.0	5.0	5.0	....
Inorganic salts	6.0	6.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	....
Ryzamin B:										
No. 1	5.0	5.0	....	....	....	....	....	....	....	....
No. 2	....	....	5.0	5.0	5.0	5.0	5.0	5.0	5.0	....
Liver extract (Lilly)	5.0	5.0	....	....	....	....	....	....	....	....
l(−)Cystine	....	.5	....	.5	....	....	....	....	....	....
l(+)Lysine	....	....	....	....	....	....	....	.62	....	....
l(−) Tryptophane	....	....	....	....	....	.1	....	....	....	....
dl-Methionine	....	....	....	....	.28	.28	....	....	....	....
Purina chow	....	....	....	....	....	....	....	....	....	100

[1] Diet 1, basal cystine; 1a, basal cystine plus cystine; 2, basal cystine, new; 2a, basal cystine plus cystine; 3, basal tryptophane; 3a, basal tryptophane plus tryptophane; 4, basal lysine; 4a, basal lysine plus lysine; 5, basal lysine, new; 6, Purina chow.

[2] In addition, all diets except 1, 1a, and 6, contained the following per kilogram of food: Natola, 150 mg.; choline chloride, 2.0 gm.; α-tocopherol, 150 mg.; and vitamin K (2-methyl-1, 4-naphthoquinone), 750 mg.

Weanling strain dba mice of both sexes were used. They were allowed to eat Purina dog chow until they reached a body weight of 16 gm. Then they were distributed among the 9 diet groups so that in no case was more than 1 mouse of the same sex and litter in the same group. On alternate days the mice were painted with a 0.2-percent ether solution of methylcholanthrene until 20 paintings had been applied. The painting site was changed with each application, as in previous experiments.

The tryptophane-free protein was made available by treating casein with hydrogen peroxide according to the method of Toennies (*12*). This process not only destroys the tryptophane but also the methionine and some of the cystine. By using 3 percent untreated casein and 7 percent hydrogen peroxide treated casein, a protein preparation was obtained which allowed no growth but excellent maintenance. *dl*-Methionine was added to this diet to allow for that lost during the peroxide treatment of the casein.

The restricted lysine diet was made by using 18 percent gliadin as a source of protein. During the course of the experiment it became apparent that this level of gliadin permitted the mice to grow (gain weight) slightly rather than just to maintain their weight, as in the cystine and tryptophane groups. Therefore it was necessary to add another group for comparative purposes in which the restriction in growth on the lysine diet would approach that of the cystine and tryptophane diets. This was readily accomplished by reducing the gliadin content [3] from 18 to 14 percent. With the exception of the animals on the old cystine-restricted and cystine-supplemented diets (groups 1 and 1a, table 3) as well as the group on chow (group 6), all mice received a vitamin supplement shown in table 3. Food intake and weight changes were recorded semiweekly. Each mouse was autopsied when evidence of leukemia was present grossly or when the animal died from other causes. Sections were taken from each animal, and all gross observations were followed by microscopic examination of stained sections.

The results are summarized in table 4. The leukemias observed were similar in gross and microscopic appearance to those previously described in dilute brown mice painted with methylcholanthrene.

The mice that ingested the old cystine-restricted diet (group 1) showed an incidence of 27.5 percent leukemia as compared with 17.1 percent previously

[3] With the group of mice on the 14-percent gliadin diets, an additional group of mice on the high-cystine diet was used as a control.

TABLE 4.—*Effect of diet on response of strain dba mice to methylcholanthrene*

Diet [1]	Effective number of mice	Mice without neoplasm	Mice with leukemia			Mice with sclerosis of aorta		
			Number	Percent	Mean latent period	Number	Percent	Mean latent period
		Number			*Days*			*Days*
1	40	29	11	27.5	131	26	65.0	106
1a	38	3	35	92.1	106	1	2.6	68
2	40	19	22	55.0	113	21	52.5	121
2a	38	3	35	92.1	97	0	0	.....
3	39	6	33	84.6	136	3	7.9	128
3a	40	5	35	87.5	91	2	5.0	100
4	39	4	35	89.9	124	0	0	.....
4a	40	4	36	90.0	110	0	0	.....
5	38	5	33	86.3	105	0	0	.....
6	38	3	35	92.1	119	0	0	.....

[1] See table 3 for description of diets.

reported (*7*). The animals on the old cystine-restricted diet supplemented with 0.5 percent *l*-cystine (group 1a) showed a 92.1-percent incidence of leukemia which was the same as previously reported. The mean latent period for the occurrence of leukemia in these two groups was 131 and 106 days, respectively.

Groups 2 and 2a show the results obtained from animals on a diet similar to that of groups 1 and 1a. However, these animals received Ryzamin B No. 2 and other vitamin supplements instead of liver extract. The incidence of leukemia in group 2 was higher (55 percent) but considerably less than in the cystine-supplemented group (92.1 percent). The mean latent periods were 113 and 97 days, respectively. A high incidence of sclerosis of the aorta was noted in the animals in group 1 which was observed previously (*7*). The mean latent period for the appearance of sclerosis was 106 days. Hence some of these animals might have developed sclerosis before leukemia would be observed. The incidence of sclerosis in group 2 was 52.5 percent, and the mean latent period was 121 days. In this group, the mean latent period of sclerosis was greater than that of the mice developing leukemia so that leukemia could have developed before the onset of sclerosis.

Groups 3 and 3a, the tryptophane-restricted and tryptophane-supplemented animals, respectively, showed approximately the same incidence of leukemia, which was high in both groups.

Groups 4 and 4a, the lysine-restricted and lysine-supplemented animals, showed a high incidence of leukemia and about the same as the control animals on the chow diet.

Group 5 is the extra lysine-restricted group. This group was more restricted in lysine than was group 4 (14 instead of 18 percent gliadin). This group was added, since group 4 with 18 percent gliadin allowed a slight gain in weight and was not comparable with groups 2 and 3. Group 5 also showed a high incidence of leukemia, it being in the same range as group 3. The incidence of leukemia in group 2 is approximately half that found in groups 3, 4, and 5. The control animals (group 6) ingesting chow showed an incidence of 92.1 percent leukemia which compares favorably with previous reports (*8*).

The animals ingesting the restricted-cystine, lysine, and tryptophane diets showed an average weight change during the first 60 days of the experiment of −0.2, −0.4, and 0.0 gm., respectively, while the animals ingesting the same diets supplemented with cystine, lysine, and tryptophane showed an average weight change of +4.5, +6.2, and +5.3 gm., respectively.

These results seem to indicate that the induction of leukemia in strain dba mice by painting with methylcholanthrene can be markedly influenced by the restriction of cystine in the diet, whereas similar restrictions of the essential amino acids lysine or tryptophane have no apparent effect. A similar reduction in leukemia has been produced by caloric restriction (*8*). It is true that the mice on the restricted diets consumed less food per animal, but based on a unit of body weight, the food consumption of the restricted and supplemented groups was equivalent. Furthermore, if one considers this as a possible effect produced by caloric restriction, then it is difficult to rationalize the marked decrease in leukemia in the cystine-restricted group and not in the lysine-restricted and tryptophane-restricted groups.

SUMMARY

A comparative study was made of the effect of the restriction of cystine, lysine, and tryptophane, respectively, on methylcholanthrene-induced leukemia in strain dba mice.

Each of the diets employed was so restricted in one of the foregoing amino acids that growth of young mice was prohibited, but indefinite maintenance was possible. The same diets, each supplemented by the amino acid in which it was deficient, permitted good growth.

There was no significant decrease in the incidence of leukemia among the mice on diets restricted in either lysine or tryptophane.

There was a reduction in the incidence of leukemia from 92.1 percent for the control group to 55 percent in the group of mice whose diet was restricted in cystine.

The data indicate that under the conditions of this experiment, cystine played a role in the development of leukemia not associated with its properties as an essential amino acid for growth but with some other attribute not yet determined.

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ABSENCE OF EFFECT OF URETHANE ON THE LATENT PERIOD OF TUMORS INDUCED WITH SUBCUTANEOUS INJECTIONS OF THE AGENT OF CHICKEN TUMOR I[1]

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INTRODUCTION

Urethane (ethyl carbamate) (*1–4*), like carcinogenic tars or hydrocarbons (*5, 6, 7*), and X- or γ radiation (*8, 9*), has been found to hasten the appearance of lung tumors, as well as to increase their frequency in inbred strains of mice, in which pulmonary adenomas develop spontaneously. Unlike carcinogenic agents of the other types, however, urethane appears to be unique in that it elicits neoplastic changes only in pulmonary tissue and only of a type similar to that which occurs spontaneously. The question may be raised whether the action of urethane, in spite of its tissue specificity, is direct as in the case of other chemical carcinogens, or whether it is indirect and effective only in conjunction with some other agent or factor, which possesses primary carcinogenic potentialities, such as that responsible for spontaneous pulmonary tumors. The factor or factors involved in spontaneous pulmonary tumor development are at present unknown, but it would seem of value to learn whether the action of any of the known carcinogenic agents can be enhanced by the administration of urethane. The present investigation was for the purpose of testing this possibility in the instance of a virus-type carcinogen, namely, the agent of chicken tumor I (*10*).

MATERIALS AND METHODS

Tumor Agent

A suspension of partially purified tumor agent was prepared from frozen tumor tissue (−50° to −60° C.) by extraction and differential centrifugation in essentially the same manner as that described by Claude (*11, 12*). The twice-sedimented and washed agent was resuspended in phosphate buffer pH 7.0, 0.005M. The same type of buffer was used as diluent in the preparation of serial tenfold dilutions of the agent suspension; no protective serum was added as in previous studies (*13, 14*). Concentrations of agent per inoculum are expressed in terms of logarithm of the number of grams of tumor tissue originally extracted. The volume of the inoculum, 0.2 cc., was constant for all concentrations of agent and for all inoculation sites.

Chickens

The chickens were of the New Hampshire Red breed and were hatched and raised in this laboratory. They were 2 to 4 weeks old at the time of inoculation and were assigned at random, without regard to sex, to the various dose groups of the two experimental series.

Urethane

A stock solution of 10 percent urethane in distilled water was used for all injections. It was filtered through a bacteria-tight filter and transferred to sterile, stoppered, serum bottles prior to use in the experiment. The dose of urethane was proportional to body weight, and each dose was regulated by varying the quantity of solution injected.

EXPERIMENTAL PROCEDURE

The experiment was designed so that 2 groups of chickens, each treated identically with a common preparation of tumor agent but only 1 treated with urethane, could be compared with respect to their latent-period responses. Each group, which was composed of 36 chickens, was subdivided into 6 dose groups of 6 chickens each, and parallel dose groups in each series were injected with successive tenfold dilutions of tumor agent. All chickens in a given dose group received subcutaneous inoculations of the same dose of agent at 4 different sites, in both wings and in both sides of the breast.

Chickens of the group that received urethane were weighed individually, and the quantity of solution required to give the desired dosage of urethane was determined in each case. In preliminary studies on a few chickens, a dosage of 0.5 mg. per gram of body weight was found to be just below the narcotizing level and was decided upon for use in the experiment. The quantity of 10 percent urethane solution required to give this dosage was injected intraperitoneally for four successive days beginning on the day following inoculation with tumor agent. A period of 6 days then followed, during which no urethane was injected, because some of the birds appeared ill (one was killed immediately for examination, and two died a day later). After the 6-day rest period, daily injections of urethane were resumed in all remaining birds in which tumors had not developed, but at a lower dosage level, i. e., 0.2 mg. per gram per day. The injections were continued in each case until a tumor appeared or until the end of the experiment. The total dose of urethane varied, therefore, in different birds and was much greater in those which received the lower doses of tumor agent and in which tumors developed after longer

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latent intervals. The total quantities of urethane administered to individuals during the course of the experiment varied from 2.0 to 20.3 mg. per gram of body weight. A single injection of 1.0 mg. per gram has been observed to enhance the development of lung tumors in mice (*2*).

As in previous studies on the latent-period response (*13*, *14*), the various site responses on each chicken were averaged to obtain the chicken response, and all latent periods were converted to their reciprocals (× 100) for the purpose of statistical analysis. The latent periods were estimated as previously described (*15*). Analyses of the data followed the principles outlined in earlier publications (*13*, *14*). Tests for statistical significance were carried out by analysis of variance (*16*, *17*, *18*).

RESULTS

In the following experiment, 2 groups of 36 chickens were compared with respect to their latent-period responses to subcutaneous injections of the agent of chicken tumor I. The first, or control group, received tumor agent only, whereas the second, or urethane group, received intraperitoneal injections of urethane in addition to tumor agent. The details are given in a preceding section. The experiment was designed so that linear dose-response curves could be employed to characterize the group responses. The individual chicken responses and the means for the various dose groups of the two series are given in table 1. Figure 1 shows the dose-group mean responses plotted against logarithm of dosage of tumor agent. The trend of the plotted points indicated a curvature, or flattening of the dose-response curve at the higher dose levels in both experimental series. As in a previous experiment (*14*, *experiment 2*), this result was interpreted as indicating that a biological limit was reached or approached at the higher dosage levels. This interpretation was further supported by analyses of variance which showed highly significant deviations from linear regressions when all dose groups were included in the analyses.

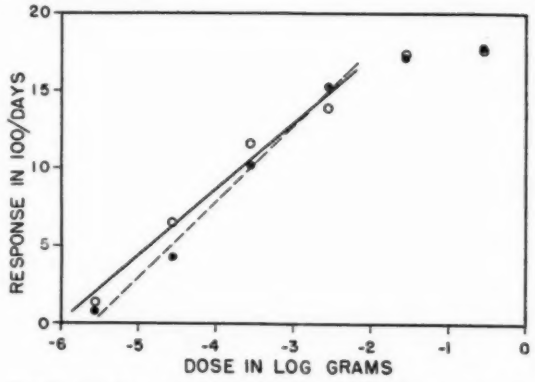

FIGURE 1.—Dose-response curves; control group, open circles and solid line; urethane-treated group, closed circles and broken line.

TABLE 1.—*Individual and group responses*

Experimental group	Dose in log grams per inoculum	−5.553	−4.553	−3.553	−2.553	−1.553	−0.553
Control	Responses in 100/days of individual chickens	0.683	1.967	13.100	12.467	15.733	18.400
		0	6.900	12.383	14.950	18.400	16.800
		0	7.100	9.600	14.033	17.900	18.400
		3.117	7.383	12.283	14.017	17.900	17.900
		2.817	6.300	12.400	14.950	17.900	16.900
		1.767	9.467	10.183	13.417	16.700	17.400
	Dose-group mean response	1.3972	6.5194	11.6583	13.9722	17.4222	17.6330
Urethane treated	Responses in 100/days of individual chickens	2.567	4.183	8.933	14.450	17.300	18.400
		0	7.467	12.600	15.733	17.900	17.400
		[2]	0	12.117	13.633	[1]	[2]
		.683	5.617	11.567	15.800	16.583	18.400
		0	8.317	2.450	16.800	17.083	16.800
		1.767	0	13.650	[1]	17.300	17.400
	Dose-group mean response	0.8361	4.2639	10.2194	15.2833	17.2333	17.6800

[1] Died from urethane injections, sixth day of experiment.
[2] Ill from urethane injections, killed for examination on fifth day.

Since the group responses for the two highest doses (−0.553 and −1.553 log grams) appeared to curve away from the trends of the remaining points, these groups were omitted, and the data were reanalyzed. Analyses of variance of the restricted data are shown in table 2 and the computed regression lines in figure 1. The slopes of the regression lines are unusually steep as compared with previous results (*13*); this phenomenon is thought to be due to the absence of protective serum in the diluent used in the preparation of serial dilutions of agent, since a similar result in the absence of protective serum was observed by Gottschalk (*19*) in studies on another chicken-tumor agent. The results in table 2 indicate that linear dose-response curves adequately describe the

restricted data and that they may be employed, as previously suggested (*13*, *14*), in comparing the responses of the two experimental groups. The comparison of control and urethane-treated groups by analysis of variance is summarized in table 3. The slight differences between slopes and positions of the regression lines are not significant statistically. Since the number of chickens in the significant dose range in each of the 2 experimental groups was relatively small, 24, small differences between their responses would not be detected in this experiment. However, the results are entirely adequate to rule out any enhancing effect of urethane on the latent period of tumors induced with subcutaneous injections of the agent of chicken tumor I comparable with the effect on lung tumors of mice; actually, the latent periods of the urethane-treated chickens were slightly though not significantly longer than those of the controls in this particular experiment.

TABLE 2.—*Analysis of variance; restricted data of separate experimental groups*

Variation	Control group				Urethane-treated group			
	Sum of squares	Degrees of freedom	Mean square	F	Sum of squares	Degrees of freedom	Mean square	F
Between dose-groups	565.4456	3	188.4819	[1] 68.963	675.7961	3	225.2654	[1] 26.433
Linear regression	551.2179	1	551.2179	[1] 201.682	668.3523	1	668.3523	[1] 78.427
Deviation from linear regression	14.2277	2	7.1138	[2] 2.603	7.4438	2	3.7219	[2] .437
Within dose-groups (experimental error)	54.6622	20	2.7331		161.9185	19	8.5220	
Total	620.1078	23			837.7146	22		
Correction	1,688.1253	1			1,232.0058	1		

[1] Significant at 1-percent level.
[2] Not significant.

TABLE 3.—*Analysis of variance; combined restricted data of control of urethane-treated groups*

Variation	Sum of squares	Degree of freedom	Mean square	F
Between positions of regression lines	6.9322	1	6.9322	[1] 1.280
Effect of combined slope	1,213.8624	1	1,213.8624	[2] 224.047
Between slopes of regression lines	5.7078	1	5.7078	[1] 1.054
Error (deviation from linear regression)	21.6715	4	5.4179	

[1] Not significant
[2] Significant at 1-percent level.

SUMMARY

In a single statistically controlled experiment involving a total of 48 chickens in the effective dose range, the effect of intraperitoneally administered urethane (ethyl carbamate) on the latent period of tumors induced with subcutaneous injections of the agent of chicken tumor I (Rous sarcoma) was studied. No significant difference was observed between the responses of control and urethane-treated chickens. The ability to react with the agent of chicken tumor I to enhance its activity does not appear to be a property of urethane.

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DEVELOPMENT OF SARCOMA AT SITE OF SERIAL TRANSPLANTATION OF PULMONARY TUMORS IN INBRED MICE [1]

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INTRODUCTION

The first report of sarcoma developing at the site of transplantation of a spontaneous adenocarcinoma of the mouse was made by Ehrlich and Apolant in 1905 (*1–6*). This phenomenon was noted in the tenth generation of transplantation, the growth containing both carcinomatous and sarcomatous areas. By the fourteenth generation all traces of the carcinoma had disappeared, and the tumor appeared to be a pure sarcoma and remained so until the twenty-sixth generation when transplantation was discontinued. The report of this observation occasioned considerable controversy among workers in the field of experimental oncology. Many doubted the accuracy of the histologic diagnosis, and there was and still is no satisfactory explanation for the mechanism of such a change. Bashford (*7*), for instance, believed that the possibility that this change represented a granulomatous rather than a neoplastic process had not been satisfactorily ruled out. A similar observation by Lewin (*8*), made during the transplantation of a carcinoma of the mammary gland in a rat, was likewise criticized on the ground that the "sarcomatous" lesion was merely granulation tissue.

However, the original observations of Ehrlich and Apolant were essentially confirmed by several investigators when Loeb (*9–11*), Haaland (*12, 13*), Liepmann (*14*), Lewin (*8*), Stahr (*15*), Russell (*16, 17*), and Marie and Clunet (*18*) independently described similar changes in the course of transplantation of epithelial tumors, chiefly of mammary-gland origin, in mice and rats. In 1917 Woglom (*19*) also observed a sarcomatous change following serial transplantation of a carcinoma but in addition noted that this change was reversible, or at least that some of his strains had lost the power of evoking sarcomatous transformation. The observation that such a profound change could occur in the histologic character of a transplantable tumor was still accepted with great reservation in 1916, as is indicated by Ewing (*20*). After discussing the subject and offering criticisms, he stated "The writer would not deny its occurrence but merely asks for more evidence." He also stated his belief that preparations of Woglom's material (*19*) which he had examined indicated that the "sarcomatous" change was due merely to the occurrence of spindle-shaped epithelial cells.

It is true that many of the figures purporting to illustrate this histologic change are not entirely convincing when judged by present-day standards, although in many cases the detailed descriptions of histologic features and clinical behavior argued strongly for the development of sarcoma in at least some of these growths.

The subject received further attention in 1945 when Ludford and Barlow (*21*), working with tissue-culture preparations, demonstrated that tumors obtained after repeated transplantations of mammary carcinomas of high-cancer-strain mice and presenting the histologic structure of sarcomas, exhibited in vitro the growth characteristics of typical sarcomas. They were essentially similar to sarcomas induced by the subcutaneous injection of carcinogenic compounds, and they differed profoundly from the mammary carcinomas in the stromas of which they were believed to have originated.

Andervont (*22, 23*) reported a sarcomatous change in 3 of 7 primary pulmonary tumors arising in and transplanted serially into strain A mice. We have had the opportunity of making histologic studies of these tumors and of 15 additional primary lung tumors induced or arising spontaneously in several inbred strains of mice. During repeated transplantations of 11 of these 22 tumors, a change in the histologic character of the growths occurred which we have demonstrated as an actual transition to fibrosarcoma.[3]

MATERIALS AND METHODS

Twenty-one of the twenty-two primary tumors of the lung were induced in the following strains of mice: 13 in strain A; 6 in strain C; and 2 in strain C3H. The additional tumor, series 11, represents a line transplanted from a spontaneous tumor of the lung arising in a mouse of strain A 12 months of age. The induced tumors of the lung arose in mice injected subcutaneously with 1,2,5,6-dibenzanthracene incorporated in cholesterol pellets or in various vehicles, as lard solution and horse-serum dispersion. The dose varied from 0.001 percent to 0.8 mg.

The primary tumors were dissected free of the lung and then divided; a portion was saved for histologic study, and the remainder was transplanted. The tumor fragments were introduced,

[1] Received for publication December 10, 1946.
[2] Formerly research fellow.

[3] A preliminary report of this study was previously presented. Stewart, H. L., Grady, H. G., and Andervont, H. B.: Observations on the histologic changes in transplanted pulmonary tumors of strain A mice. 3d Internat. Cancer Congr., pp. 147–148. Atlantic City, September 11–15, 1939.

by means of trocar and cannula, into the subcutaneous tissue of the right axilla of mice of the same strain as the animal in which the tumor originated. The rate of growth of the transplanted tissue was not accurately measured; but the intervals between successive transplantations usually became shorter, so that by the second or third generation transplantation could be carried out at 3-to-4-week intervals as compared with the 2-to-3-month interval which was the rule for the first transplantations. The transplanted tumors were removed with aseptic precautions and again divided for histologic study and further transplantation.

Tissues were fixed in Tellyesniczky's [4] or acid Zenker's fluid or dilute formaldehyde solution (U. S. P. 1-10), embedded in paraffin, and stained with hematoxylin and eosin, van Gieson's stain for collagen, Foot's modification of the Bielschowsky technique for reticulum, and phosphotungstic acid-hematoxylin for fibroglia fibrils.

Primary Pulmonary Tumors

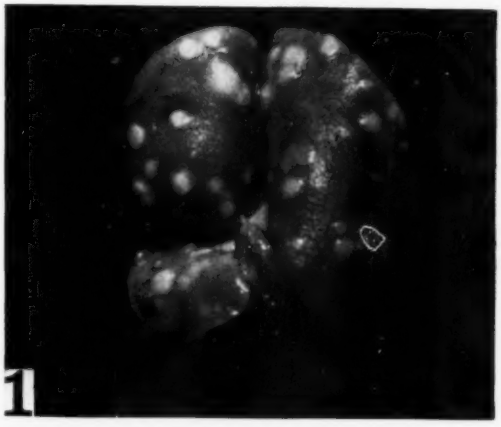
Grossly, the pulmonary tumors selected for transplantation were round, sharply circumscribed, pearly white nodules projecting slightly above the pleural surface of the lung (fig. 1). They were usually multiple, although the original tumor of series 11 was a solitary spontaneous growth. Microscopically, they were all pure acinous growths, generally composed of thin connective tissue stroma covered by cuboidal or columnar tumor cells (fig. 2). Occasionally, the cells in a few areas grew in closely packed, irregular columns and in a few instances in solid sheets of the same type of cell. The nuclei varied slightly in depth of staining; mitotic figures were few except in two tumors, strains Nos. 13 and 16. In one instance, in tumor strain No. 15, large, round, acidophilic, nuclear inclusions were observed. The stroma was usually scanty and consisted of delicate collagenous bundles containing a few mature fibroblasts. The small amount of reticulum present was confined to these cores and did not enter into intimate contact with the covering epithelium (fig. 3). Blood vessels were few and were usually of capillary structure and located within the cores of the papillae. The tumors were adjacent to the pleura and unencapsulated, although the surrounding pulmonary tissue was often somewhat compressed. In several instances there appeared to be a confluence of two or perhaps three primary tumors. This was indicated by partial separation of the growths and by minor differences in architectural arrangement. In general all the tumors studied exhibited characteristics similar to the well-known primary tumor of the lung (Grady and Stewart (*24*)).

[4] Two parts formol, 20 parts 70-percent alcohol, and 1 part acetic acid.

Transplanted Tumors

Gross Description

The transplanted growths when selected for transfer varied in size depending largely upon the growth interval. They often attained dimensions as large as 25 × 15 mm. if allowed to grow for a sufficient period. They were circumscribed and more or less encapsulated. The smaller growths



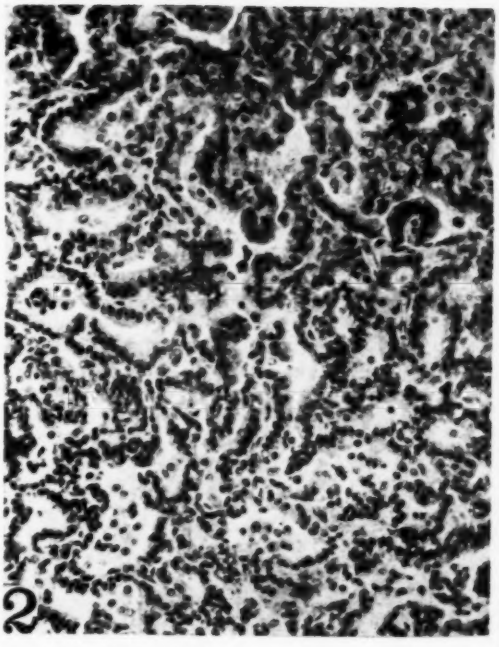
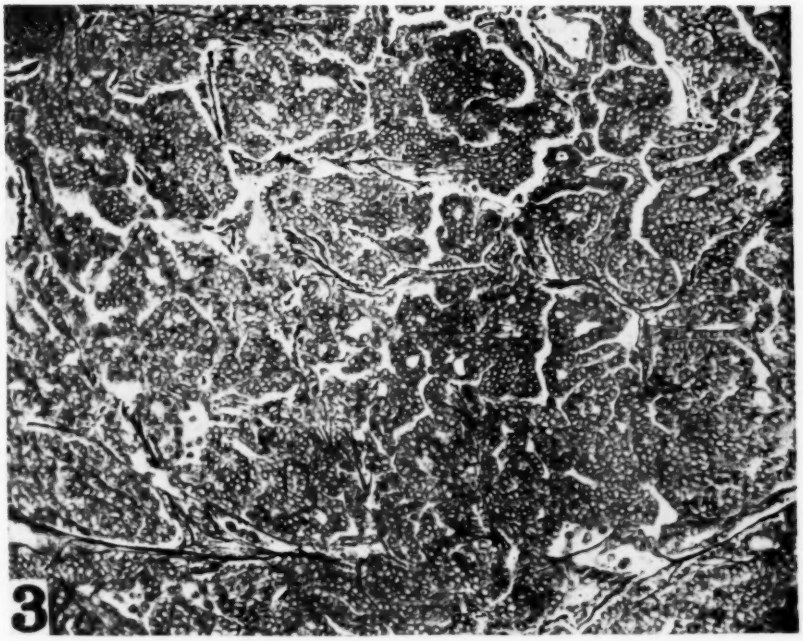


Figure 1.—Induced pulmonary tumors. Photograph of gross specimen. × 3.8.

Figure 2.—Primary tumor of the lung, showing papillary glandular arrangement. Line 21. × 200.



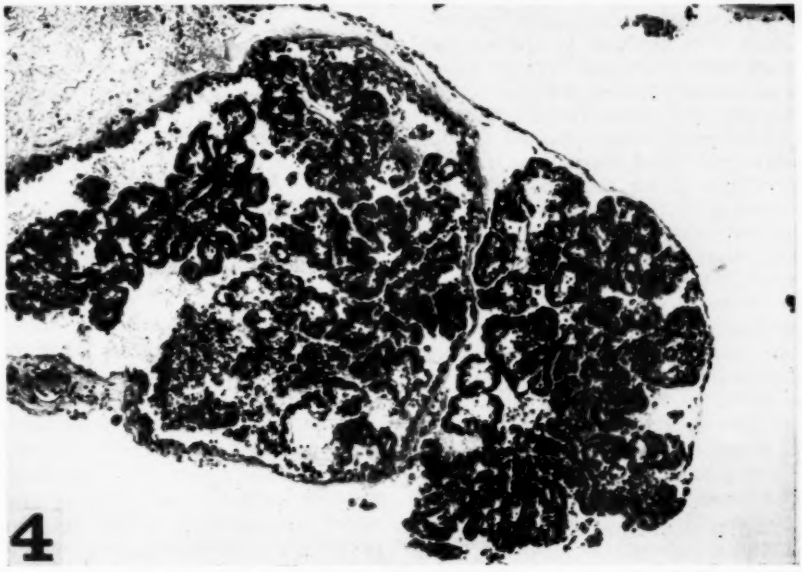


FIGURE 3.—Reticulum in primary pulmonary tumor. Line 2. × 140.

FIGURE 4.—First-generation transplant showing glandular epithelial arrangement. Line 20. × 80.

were composed of firm, gray or pinkish tissue and were more firmly adherent to the overlying skin than to the chest wall. As they grew larger, many of the epithelial tumors formed central cystlike cavities which were filled with either clear or blood-stained fluid. The lining tissue of these cavities was sometimes smooth and sometimes covered with friable mural nodules. Cystic changes were not found in the transplanted growths which proved to be of pure sarcomatous nature. The skin overlying the larger growths was frequently ulcerated and covered with a firm crust. In those transplanted tumors in which bone developed, it was not detected grossly. In another series of transplanted pulmonary tumors, however, we have seen a tumor measuring 15×15×7 mm., half of which was hard and contained gritty material, on gross examination, which proved to be bone, while the other half was composed of firm gray tissue. The tumors containing both carcinomatous and sarcomatous elements did not differ significantly in gross appearance from the purely glandular tumors.

HISTOLOGIC DESCRIPTION

It was possible to divide the tumor strains into several histologic types, and for convenience these are described separately.

Acinous carcinoma.—As long as transplanted pulmonary tumors preserved their original glandular character, they were uniform in appearance (fig. 4). They were most often papillary growths, surrounded by a mantle of connective tissue which subdivided the tumor into compartments or cystlike spaces. The tumor cells were cuboidal or columnar and closely resembled those seen in the primary tumor. They lined the walls of the glandular and cystic spaces and covered the thin cores of the papillae, usually in a single row although occasionally they were stratified, a characteristic seldom seen in the primary tumor. Very frequently there were masses of desquamated tumor cells lying free in the cystic spaces. In 15 individual transplants in 11 different tumor lines, the epithelium lining the cystic spaces or covering the papillae was flattened and elongated in focal areas. This condition is designated F in table 1. These flattened, elongated cells were either attached at one end to a basement membrane or lay parallel with the underlying connective tissue. They seemed to be found more frequently where a loosening or partial desquamation of the adjoining epithelium had taken place. Frequently these cells appeared to extend below the basement membrane and to invade the surrounding connective tissue for short distances so that it was sometimes difficult to distinguish between these flattened epithelial elements and the adjacent stromal cells. Mitotic figures in general were more numerous in tumors of this type than in the primary tumor.

The connective tissue mantle surrounding the transplant was usually thick and frequently contained inflammatory cells and granulation tissue. A few infiltrated tumor cells might also be found. The stroma of the papillae on the other hand was most often thin and usually contained a single central capillary (fig. 5). In three microscopic sections, small foci of osseous tissue were observed within the cores of the papillae (fig. 6). The connective tissue in these cores was sometimes hyalinized, and a few osteoblasts could be seen about the newly formed bone. These bony deposits appeared always within the stroma and were entirely distinct from the tumor cells. No marrow cavities were associated with the bony deposits.

Nonacinous carcinoma.—In many transplants the tumor cells, although not in acinous formation, could still be recognized as of epithelial nature. They were arranged (1) in columns of varying width and length separated by a connective tissue stroma, (2) in small, irregular nests, and (3) as relatively solid sheets.

The relative proportion of acinous and nonacinous types of tumor varied within wide limits; and within the same growth different proportions of the various nonacinous carcinoma forms 1, 2, and 3 might be found. Almost any combination of these various structural arrangements might be found in a given generation of transplantation.

When tumor cells occurred in columns, they varied moderately in shape, being sometimes oval, round, wedge-shaped, or polyhedral (figs. 7, 8, 9). The columns were usually closely packed and frequently curved, but they did not interlace as did the typical bundles of the sarcomatous tumors. The stroma between the columns varied somewhat in amount but usually was not large.

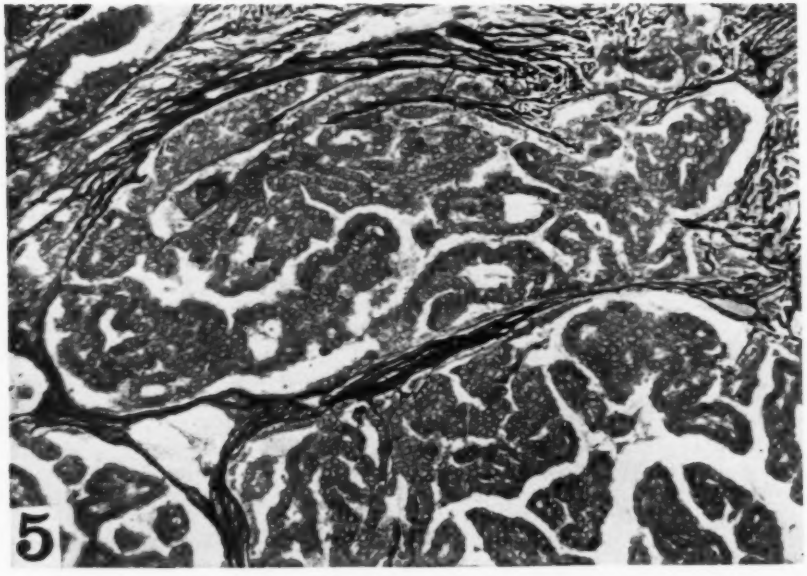
The individual cells in the nonacinous tumor had a single large darkly staining nucleus often varying considerably in size (figs. 10, 11). The cytoplasm was smooth and either acidophilic or faintly basophilic. While there were fairly wide variations in the configuration and staining properties of these cells, their resemblance to epithelial elements was generally recognizable. Mitotic figures on the average were far more numerous than in the primary tumors of the lung. In some tumors, the cells were arranged in sheets, a condition which appeared to be more common about the periphery of the transplant, and they were closely packed together without any appreciable intercellular substance. The general appearance of these areas was suggestive of pavement epithelium, but there were no intercellular spines, keratinization, or pearl formation. The nuclear structure and staining qualities of the cells did not differ

TABLE 1.—*Sequence of passages and histologic character of 22 transplanted pulmonary tumors and their 45 lines, direct and collateral*

Generation	Tumor series 1	2	3	4	5	6	7	8	9	10
0		G	G	G	G	G	G	G	G	G
1	G/A	G/F, G/F	G/SA	G	G	G/A	G/B	G	G	G
2	G/A	G/F, G/F	G/GA, A/G, A/S		G	G/AS		S/AG	GA	G
3	G/A	G/AF	A/G			G/AS		S/A	GA	G
4	G/A, G/A	G/AF, G/AF, G	A/G			GAS		S/A	G/A	G
5	G/AS	G/A	A			GAS		S/A	A/G	G
6	A/SG	G/A	A			S/AG		S/A	A/SG	G
7	A/GS, A/G	A/SG	A/S							G
8	A/G	A/S	A							G/F
9	A/G	A/S	A							G/F
10	A/G	A/GS	A							G
11		A/GS	A							G
12		A/GS								G
13		A/GS								G
17		S/A								
49	A/G									

Generation	Tumor series 11	12	13	14	15	16	17	18	19	20	21	22
0	G	G	G	G	G	G	G			G	G	G
1	G, G/B	G/F, G/F, G	G	G, G/AS	G	GA	G/A	S/GA	G/FA	G	G/ASB	G
2	AS/G	G/A, G/A	G	G/AS	A/G	GA	G/A	S/A	A/GS	A/GS	G/B, G/ASB, G, G/AS	
3	S, S	G/A, G/AF		G/ASF	G/A	GA	G/A	S	S/A, A, S/G	G/S	S/AG, SAG, G, G/AB, S/AG	
4	S	G/AF		S/A	A/G	GA	G/A	S	S/A	S/A	S/GA	
5	S			S	A/G	A/G	G/A	S	S/A	S/A	S/AG	
6	S			S	G/A	A	G/A		S/A	S/A	S/AG	
7	S				G/A			S	S/A	S/A	S/A	
8	S							S	S/A	S/A	S/A	
9								S	S/A	S/A	S	
10								S	S/A	S/A	S/A	

S, sarcoma; G, acinous carcinoma; A, nonacinous carcinoma; B, bone; and F, flattened epithelium.



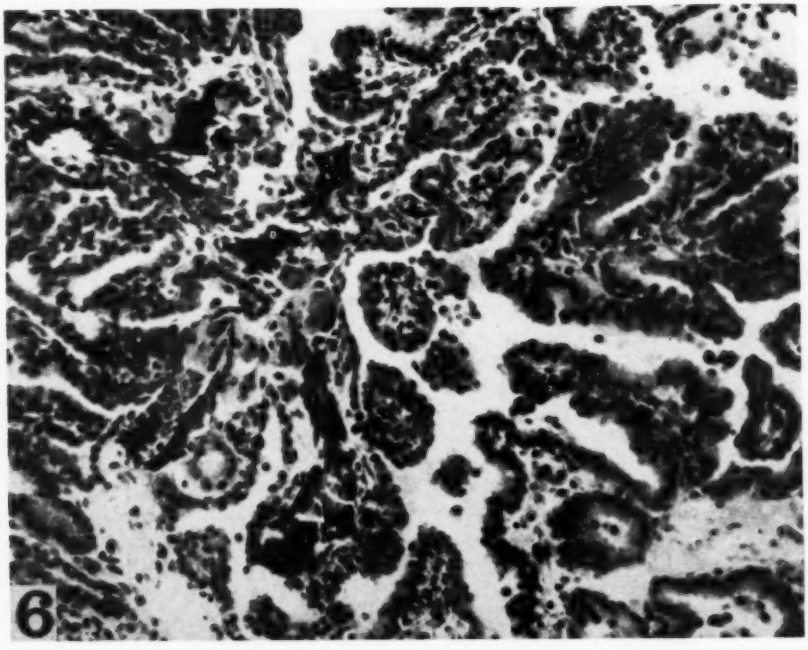
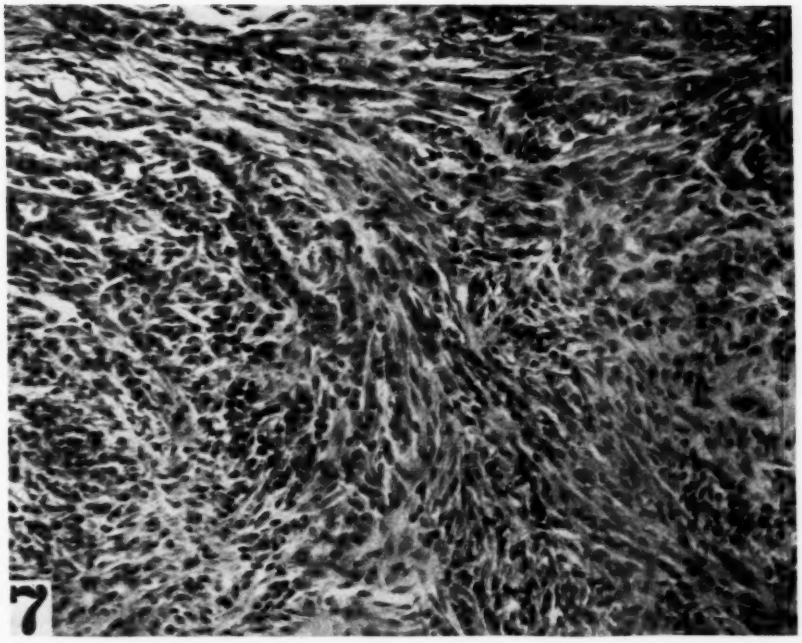


FIGURE 5.—Reticulum in transplanted tumor showing glandular epithelial arrangement. First-generation transplant line 2. × 200.

FIGURE 6.—Second-generation transplant showing small deposits of bone in the stroma. Line 21. × 200.



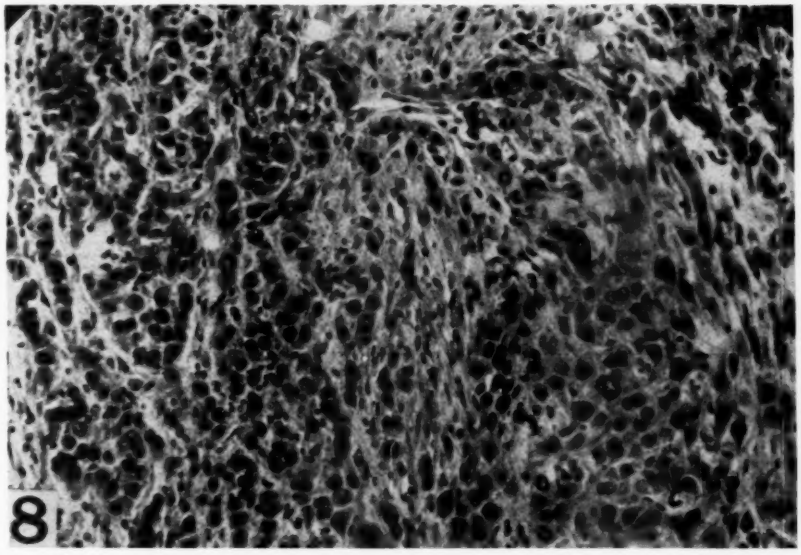
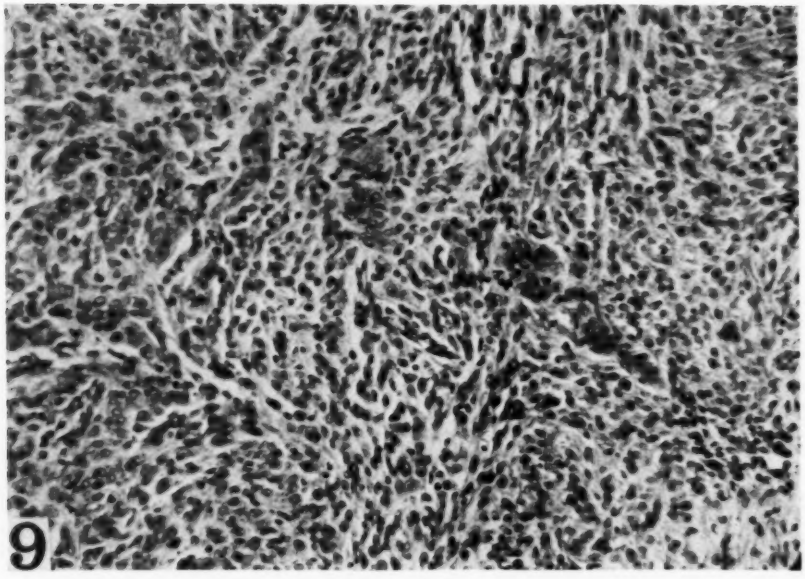
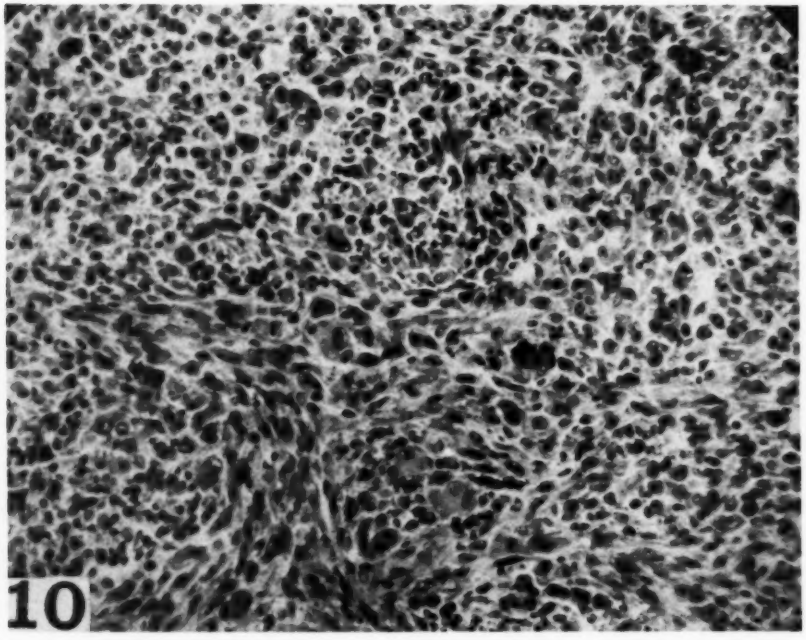


FIGURE 7.—Second-generation transplant. The epithelial component of the tumor is recognizable as cords and nests of cells; many spindle cells are present tending to form narrow bands. Line 16. × 200.

FIGURE 8.—Sixth-generation transplant. Tumor cells are predominantly epithelial in type, occurring in nests and cords, with considerable variation in size, shape, and staining of these cells. Many atypical spindle cells are present. Line 2. × 200.





FIGURES 9–10.—Fourth-generation transplant. Figure 9, nests of epithelial cells present in an area of the tumor occupied principally by spindle cells. The latter stain deeply and show numerous mitoses. Line 6. × 200. Figure 10, extremely cellular tumor; individual cells vary considerably in size, shape, and staining and are in rather haphazard formation. In the lower half of the field, they show a tendency to form a bundle. Line 20. × 200.

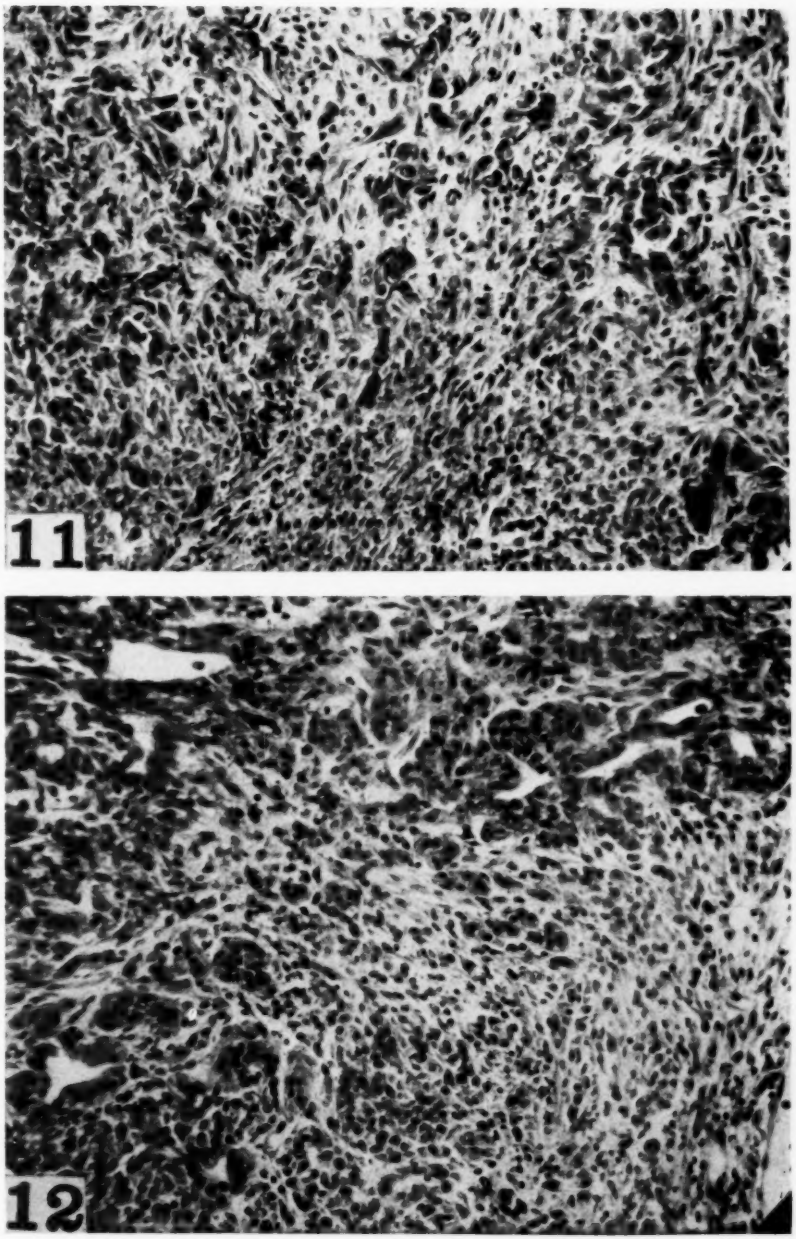


FIGURE 11.—Second-generation transplant. There is considerable variation in cell type; some recognizable epithelial cells are in nests and in collapsing acini. Much of the tumor is composed of spindle cells. Line 20. × 200.

FIGURE 12.—Second-generation transplant line 16. Part of the tumor is composed of acini and nests of epithelial cells. To the right are many atypical spindle cells. × 200.

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appreciably from those in columns, although the cytoplasm on occasion stained palely and sometimes contained hyaline droplets.

In transplants which possessed an abundant stroma, tumor cells were sometimes observed in small, irregular clumps and nests of imperfectly formed acini usually embedded in dense connective tissue (figs. 12–15). The individual cells varied considerably in shape but were generally recognizable as epithelial in nature.

In lines 19 and 20, tumor giant cells were seen. They were more frequent in the peripheral areas of the tumor, and there was little intercellular substance. Most of the nuclei were huge, with a prominent nuclear membrane and large chromatin masses set in an otherwise clear area. The nuclei were lobated and deeply indented, so that it was often difficult to decide whether the nuclei were multiple or multilobed. The cytoplasm was abundant, basophilic, and most often smooth although in some instances there were fine vacuoles present. The shape of the cells was inconstant and ranged from round to polyhedral or elongated forms. Mitotic figures were numerous.

Blood vessels were infrequent and were usually of small caliber with relatively well developed walls. However, in a few sections large numbers of red blood cells were seen between thin bundles of tumor cells, and it was difficult to determine whether these were within thin-walled blood vessels running parallel to the tumor bundles or whether they represented small areas of hemorrhage. Infrequently blood vessels of an irregular sinusoidal type were seen coursing about in such devious fashion as to isolate, partially or completely, small islands of tumor cells (fig. 16). Occasionally blood vessels were numerous, thin-walled, and irregular.

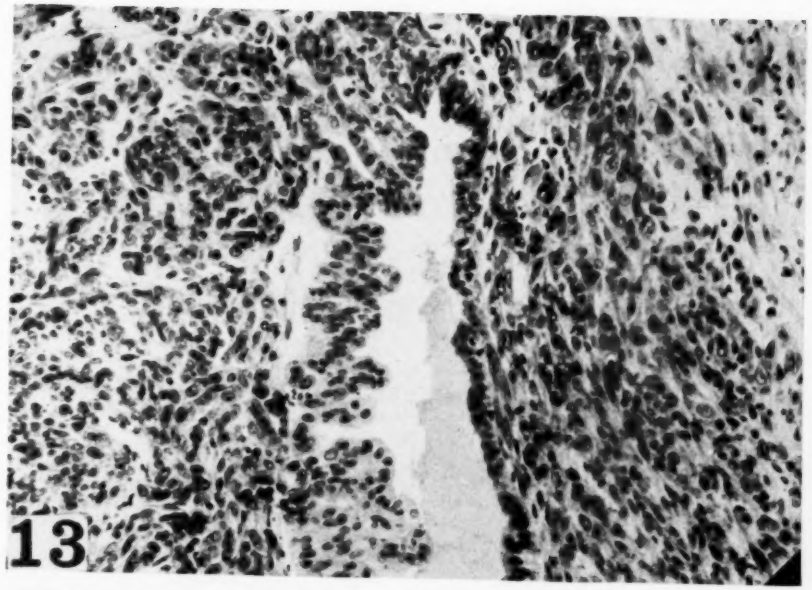
Sarcoma.—The structural features of the transplanted tumors after they became sarcomatous were on the whole uniform. These growths were composed of plump bundles of elongated spindle cells which formed interweaving and interlacing patterns (figs. 17 and 18). The spindle-shaped tumor cells were usually large, with elongated, darkly staining nuclei; mitotic figures were numerous. The cytoplasm was frequently drawn out into thin, unipolar or bipolar processes, which suggested fusion with those of adjacent cells at their extremities. The van Gieson and silver preparations revealed a great increase in the amount of collagen and reticulum, as well as a profound change in distribution of these intercellular substances when compared with the epithelial growths (figs. 19, 20). The reticulum fibers formed an extremely close meshwork inclosing cells singly and in small groups and was intimately applied to the sides of the cells. In the phosphotungstic acid-hematoxylin preparations, numerous fine fibroglia fibrils were associated with the tumor cells (fig. 21).

Blood vessels were usually numerous, immature, thin-walled, and irregular in outline. They were lined partially or completely with endothelial cells, although in some areas the tumor cells seemed to border directly upon the vascular channel or to be growing into it.

Combined epithelial and sarcomatous tumors.—Table 1 shows that many individual tumors consisted of combinations of several or even all of the general histologic types which have been described, namely, the acinous and nonacinous carcinoma and sarcoma (figs. 7–16). The relative proportion of the different types also varied as may be seen in the table. The predominant type is listed as the numerator. Since it is not feasible to give detailed descriptions of all possible combinations in every one of the tumors, the description of some of the salient features of two of the mixed growths is given as a representative example.

Frequently the transplanted tumors formed papillary glandular structures consisting of a cyst-like or large glandular space in which more or less complex papillae were found. The lining cells of these spaces were of considerable interest. They were mostly recognizable as epithelial elements, but there were extreme variations in the shape of the cells. There were low cuboidal forms, and in some areas the cells became elongated and spindle-shaped and arranged parallel with the basement membrane. The tapering ends of these cells were seen to be continuous with adjacent cuboidal cells. In other instances, particularly when the glandular lining was columnar and pseudostratified, the polarity of the spindle-shaped cells was reversed, i. e., one tapering end projected toward the lumen and the other anchored at what would correspond to the basement membrane. In both types, however, examples were seen when, within a localized area of the gland space, the spindle-shaped cells extended through the basement membrane and into surrounding stroma. When this occurred, the cells were usually arranged in thin bundles of varying length, straight or curved. Sometimes they were associated with or came in close contact with sarcomatous areas. For the most part this invasion was for only limited distances into the stroma.

While the papillary cores were usually thin and the covering epithelium sharply demarcated from the connective tissue, there were a few examples in which richly cellular cores were merged with the covering epithelial cells. In these areas, the epithelial cells were often elongated and spindle-shaped and intermingled with similarly shaped cells, some of which appeared to be of stromal origin.



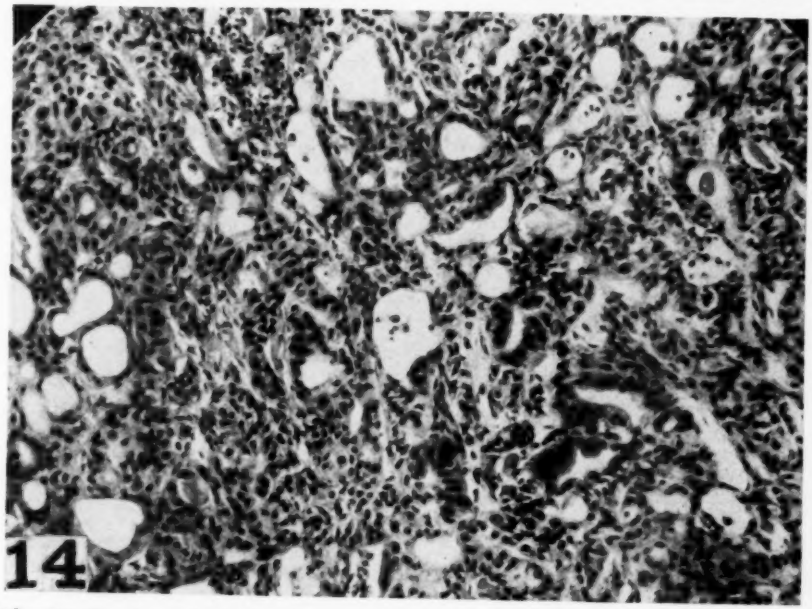
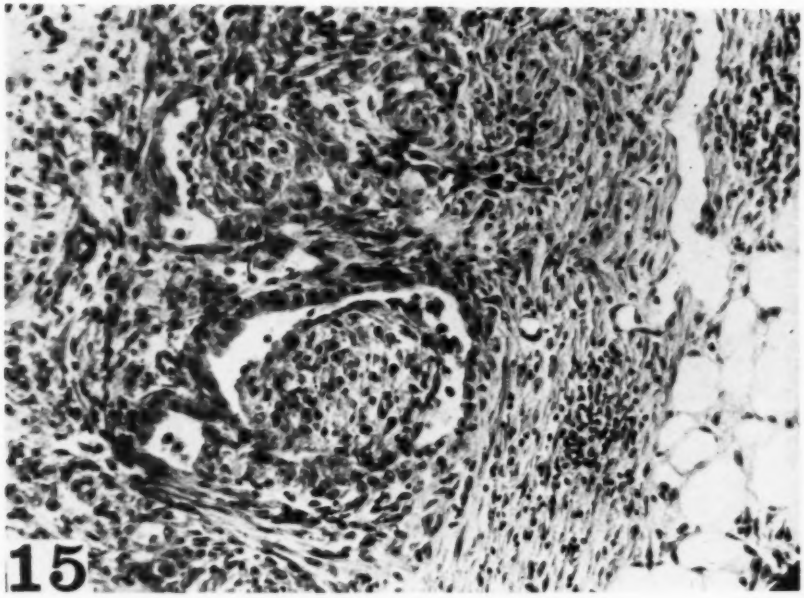


FIGURE 13.—Third-generation transplant. In the center is a large gland space lined by cuboidal and columnar cells showing a papillary arrangement on the left. Surrounding the area are nests of tumor cells which appear epithelial. Other cells are spindle-shaped. Line 21. × 200.

FIGURE 14.—Seventh-generation transplant. The lining cells of the acini vary from tall columnar to flattened; epithelial nests are present; stroma is abundant and cellular; cells are atypical. Line 15. × 200.



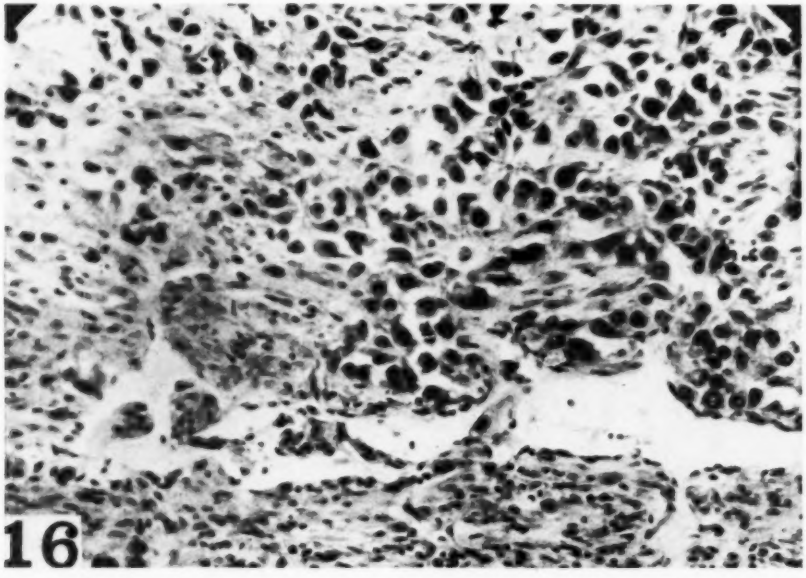


FIGURE 15.—Fifth-generation transplant. Large acinus shows invasion of its lumen by a plug of atypical spindle cells. in several areas are spidle cells becoming arranged in thin bundles. Andervont's tumor line H. × 200.

FIGURE 16.—Seventh-generation transplant. Considerable variation in the appearance of the tumor cells; many show cytoplasmic processes; some are spindle-shaped. Note formation of a large vascular space in the lower portion. Line 2. × 200.

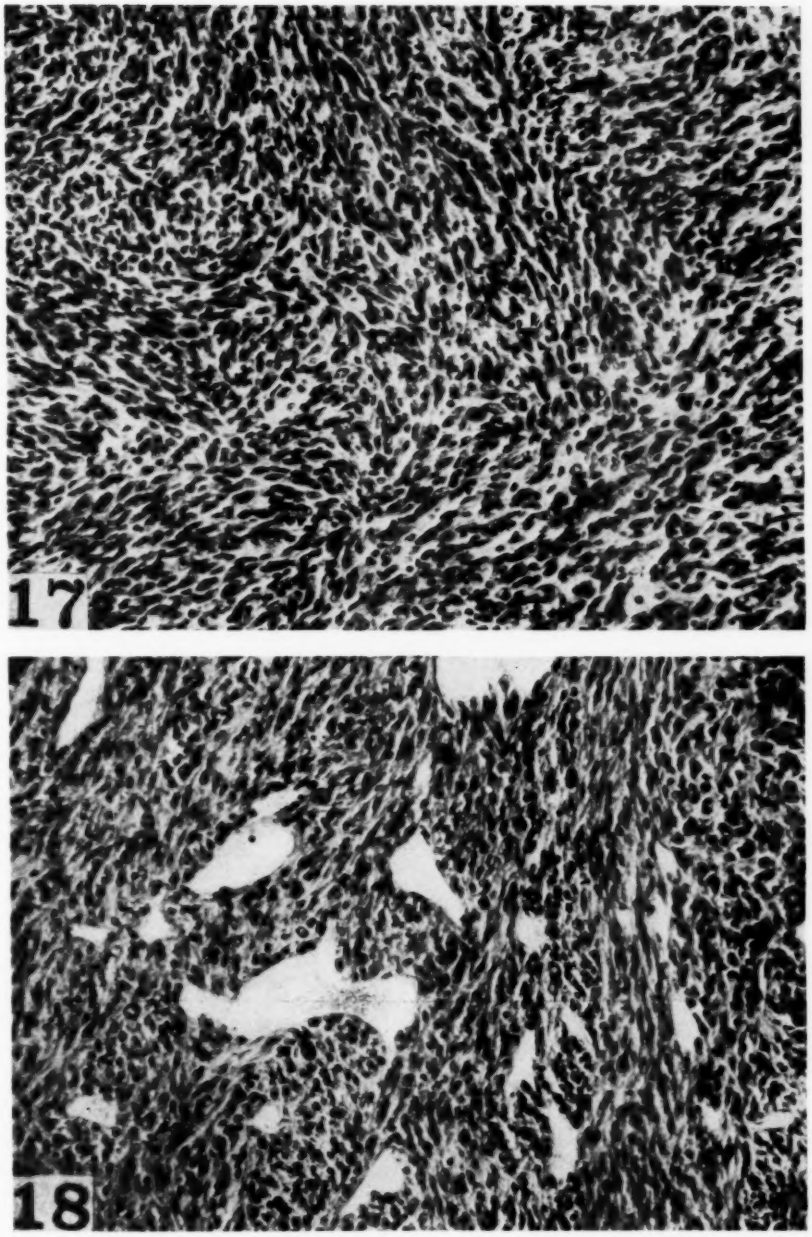
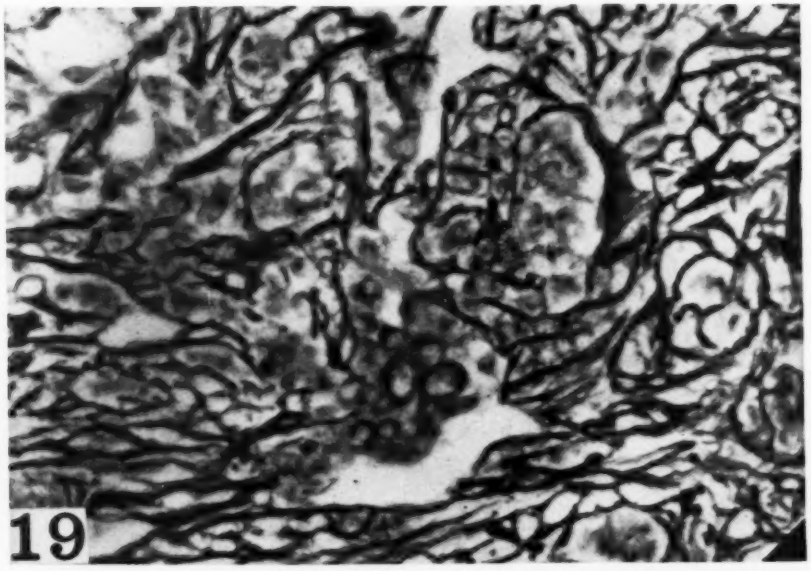


FIGURE 17.—Sixth-generation transplant. Fibrosarcoma area. Line 20. × 200.

FIGURE 18.—Ninth-generation transplant. Fibrosarcoma showing characteristic type of vascular channel. Line 21. × 200.



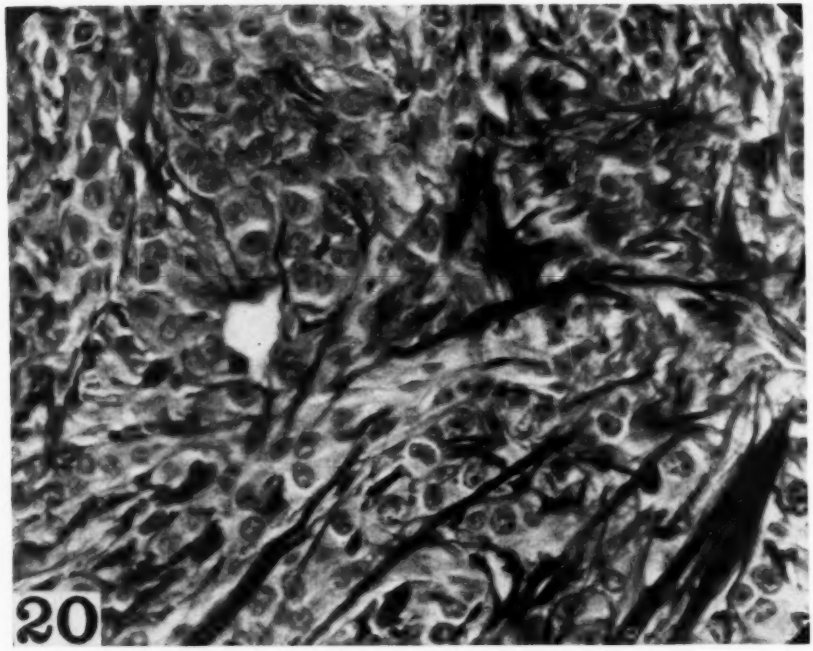


FIGURE 19.—Characteristic distribution of reticulum in fibrosarcoma. Seventh-generation transplant line 2. × 400.

FIGURE 20.—Fibrosarcoma showing distribution of collagen. Seventh-generation transplant line 2. van Gieson's connective tissue stain. × 400.

FIGURE 21.—Fibrosarcoma showing fibroglia stained with phosphotungstic acid-hematoxylin. First-generation transplant of lung tumor arising in strain A mouse No. 2, Stewart experiment 16. Transplant grown for 15 months. Fixed in acid Zenker's fluid. Circa × 1500.

Likewise, when combinations of sarcoma and nests of epithelial tumor cells were observed, variant forms of epithelial cells were not uncommon. The epithelial cells might be pear-shaped or elongated spindles, sometimes arranged in thin columns. While these thin columns and single elongated cells were often intimately associated with sarcomatous bundles, the two elements were most often distinguishable by differences in nuclear characteristics. In some instances, however, it was difficult to decide whether a given cell or group of cells was epithelial or stromal in nature.

Where a sarcoma developed following serial transplantation, it was impossible to determine the site of origin of the cell from which the sarcomatous growth was derived. Serial transplantations sometimes yielded purely sarcomatous growths, while in other lines there were all possible combinations of histologic tumor types (table 1). It is true, however, that when a sarcoma appeared in combination with glandular growth, there was a definite tendency for the acinous to give way to the nonacinous type of tumor. Less often sarcomatous growths were seen in intimate physical association with invading columns or nests of spindle-shaped epithelial cells as just described. Careful examination of this material failed to reveal convincing evidence that this phenomenon represented anything more significant than mere association of the two types of growth. Whether more conclusive transitional phases or causal relationships exist between the occurrence of spindle-shaped epithelial cells and spindle-cell sarcoma remain unanswered by this study. There are suggestive observations that the development of spindle-shaped epithelial cells is a necessary antecedent condition for the transformation of certain epithelial tumors to sarcoma.

Table 1 is arranged to illustrate the sequence of passages and the histologic character of the 22 tumors and their 45 lines, direct or collateral. Tumors Nos. 1, 2, 3, 4, 7, 9, 11, 12, 15, 18, 19, 20, 21, and 22 arose in strain A mice; tumors Nos. 5, 6, 10, 13, 14, and 17 in strain C mice; and tumors Nos. 8 and 16 in strain C3H mice. In 14 of the 22 series, the sequence of passage was direct without sublines, while in 8 a primary tumor or a transplant was divided into several portions and trans-

planted into 2 or more animals, thus giving rise to collateral lines. Likewise in several lines, some of the collaterals were discontinued while others were carried in serial passages. Since many of the transplanted growths were of mixed histologic type, it is not surprising that in tumors 1 and 3 sarcomatous areas appeared and then were not seen in later passages. Conversely, elements might disappear for a time and then reappear, e. g., acinous carcinomatous areas reappeared in passages 8, 9, and 10 of tumor 2 after having disappeared in earlier passages. In tumor 3 the first-generation passage contained a sarcomatous area and was transplanted into 2 second-generation animals. One of these contained a sarcomatous area, and this remained in subsequent transplant, after which the subline was discontinued. The other second-generation transplant failed to show any sarcoma: possibly only the sarcomatous portion was used for transplantation. Moreover, this line was carried to the eleventh generation and showed only 1 small sarcomatous area in the seventh passage. A similar change occurred in passages 8, 9, 10, and collateral 7 of tumor 1. Sarcomatous areas appeared in passages 5, 6, and 7 but disappeared thereafter.

A study of the final histologic status of the various tumors and their collateral lines shows that in 5 lines the tumor transplant had become wholly sarcomatous in type whereas in an additional 13 lines the tumor was a combination of sarcomatous and carcinomatous structure. The acinous carcinoma type was found in 8 instances, nonacinous carcinoma type in 3, and combinations of acinous and nonacinous carcinoma in 7 transplants. Flattened epithelium was seen in combination with acinous carcinoma type 7 times, and with combined acinous and nonacinous carcinoma 8 times. A total of 3 growths contained bone in the last generation maintained; 2 of these bony areas were in acinous carcinoma growths, and the third was in a combined acinous and nonacinous form. The bone formation persisted for from 1 to 3 generations.

Table 1 also shows that in eight of the last generations carried, acinous growths had been replaced by the nonacinous type following transplantation and that in three additional lines nonacinous types had been combined with the acinous. There appeared to be a tendency then for the nonacinous to replace the acinous type. In no instance in this series did a sarcoma develop unless it was associated with or preceded by a change from acinous to nonacinous type. When the sarcoma change occurred, it was seen for the most part in the early generations of transplantation, four being seen in the first generation, six in the second, and one each in the fifth, sixth, and seventh.

In the 5 instances in which the final passage was wholly sarcomatous, the sarcomatous change had been present in combination with other types for 1 generation in 2 instances, and for 2, 4, and 7 generations, respectively, in the other 3. In these cases, there seemed then to be a tendency for the sarcomatous growths to develop at the expense of the nonacinous type. In the 13 cases in which the final result was a sarcoma in combination with carcinoma, the sarcomatous areas had been present for 1 generation in 1 instance, for 2 generations in 2 instances, for 3 generations in 4 instances, and for 5, 9, and 10 generations, respectively, in 2 instances.

DISCUSSION

That the primary tumors of the mouse lung possess an epithelial pattern is clear from the studies of many investigators, including Tyzzer (*25, 26*), Slye, Holmes, and Wells (*27, 28*), Haaland (*12, 13*), Jobling (*29*), and others. More recent studies by Grady and Stewart (*24*) indicate that the induced pulmonary growths arise from alveolar lining cells. While spindle-shaped cells may be seen at certain stages in the developing pulmonary tumor when the alveolar lining cells are enlarging and are still attached to the alveolar wall, these cells ultimately take on a cuboidal appearance and become arranged as papillary adenomatous growths. The tumors consist of a thin papillary core covered by one or more layers of cuboidal or columnar tumor cells. There are frequently more solid areas, but they are clearly papillary growths which have been compressed.

As noted previously, stains for collagen and reticulum reveal a delicate stroma, which is limited to the papillary cores and which does not surround single cells or small groups of cells as in a typical fibrosarcoma. It is true that rarely in old primary lung tumors spindle-cell areas of varying size may occur, and we have observed six such growths among many thousands studied in this laboratory. Slye, Holmes, and Wells (*27, 28*), moreover, noted the occurrence of spindle cells in the metastases from primary pulmonary tumors. This indicates that although the transition from carcinoma to sarcoma, which we have described in the transplanted pulmonary tumor, may and does take place within the animal in which the tumor arose, the overwhelming majority of primary tumors of the lung in mice correspond to the type which we have described.[5]

The long-standing controversy on the nature of the alveolar lining cell, whether epithelial or mesenchymal or both, from which the primary pulmonary growths are derived, is not as yet

[5] Primary pulmonary tumors of mice histologically different from the common pulmonary tumor employed in this study have been observed by Larsen and Dunn and by Lorenz and Stewart in this Institute. Descriptions of these tumors will be reported later.

settled. The papers of Loosli (*30*), Fried (*31*), Miller (*32*), and Bremer (*33*) contain detailed discussions of this subject. There is such difference of opinion among competent embryologists that no final opinion on this point can be rendered at present. Whatever the embryologic origin or phagocytic potentialities of the alveolar lining cells prove to be, the fact remains that growths with all the recognizable structural attributes of epithelium can and do arise from the alveolar lining of the mouse lung.

The transplanted tumors which form the basis of this study have been diagnosed as fibrosarcoma because of certain morphologic features that are generally accepted as distinctive for this tumor. They include not only the spindle shape of the tumor cells but also more significantly (1) the presence of interlacing bundles of tumor cells, (2) the intimate association of collagen and reticulum with the tumor cells, and (3) the presence of fibroglia fibrils associated with these cells. Spindle or fusiform outline of tumor cells alone obviously does not establish a growth as a sarcoma, as many pathologists have noted. The occurrence of spindle-shaped epithelial cells has been repeatedly observed particularly in certain carcinomas of the bronchus, lip, and cervix in human beings. However, the spindle-shaped cells in these epithelial tumors tend to grow in more or less solid sheets and not in interlacing bundles of varying thickness. While tumor cells growing in sheets were not infrequently seen in some of these transplanted tumors, they were not spindled or fusiform. In the great majority of cases in which spindle-shaped tumor cells occurred either alone or in combination, they were seen in the form of long, thin, curving, and interlacing bundles. The amount and distribution of reticulum in the sarcomatous growths differed significantly from that seen in the primary tumors and in those transplants that retained their carcinomatous character. In the glandular tumors, whether primary or transplanted, reticulum and collagen were both relatively scanty and were concentrated largely in the cores of the papillae or in their branches. There was no evidence that the reticulum was in intimate contact with tumor cells individually. In the sarcomatous growths on the other hand a radically different picture was seen. With van Gieson's stain for collagen, the phosphotungstic acid-hematoxylin stain for fibroglia fibrils, and Foot's silver method for reticulum, there was a remarkable difference in the amount and distribution of collagen, and more especially the reticulum fibers, with the added presence of fibroglia in the sarcomas as compared with the epithelial tumors. The reticulum particularly formed a close, intricate network, which was intimately applied to small groups of sarcoma cells or more frequently completely surrounded individual cells.

The character of the blood vessels likewise differed sharply in the epithelial tumors, primary or transplanted, as compared with the sarcomas. The primary tumors had a relatively small vascular component which was represented chiefly by capillary vessels in the papillary stroma. The transplanted growths of epithelial character were similar, although the connective tissue capsule about the transplant frequently contained many thin-walled capillaries. Many of the sarcomas, however, contained within the substance of the tumor large numbers of irregular, sinusoidal channels, usually lined by endothelium but in some instances by tumor cells. These sinusoidal vessels were virtually always devoid of any more complex coat, and occasionally strands or islands of spindle-shaped tumor cells were seen projecting into the vascular lumen.

The mechanism and underlying causes of sarcoma development following serial transplantation of epithelial tumors, primary in the lung of the mouse, cannot be explained at present on the basis of our material. There are certain immediately obvious theoretical explanations which have been briefly examined, but no definite conclusion has been reached. The primary lung tumors possess a stroma which is sparse and delicate. It is conceivable that this stroma assumes neoplastic properties following transplantation and overgrows the epithelial tumor. However, nothing has been observed to indicate that this is the explanation in this series of observations. Before the days of inbred strains of mice, Bashford (*7*) and his coworkers demonstrated that the stroma of transplanted tumors is soon replaced by a stroma derived from the tissues of the new host. Ludford and Barlow (*21*) believe that if inbred strains are used, it may be expected that when a tumor is transplanted into mice of the same genetic constitution, there is increasing likelihood that the transplanted stroma elements persist since the cells of the transplanted tumor stroma are under these conditions homozygous with those of the new host. They found this to be true when transplanting mammary tumors in inbred mice.

The histologic changes occurring during sarcomatous transformation of the stroma of carcinomas were fully described by Haaland (*12*, *13*). Ludford and Barlow (*21*), working with breast tumors in high-cancer-strain mice, were able to endorse his statements that neither in the primary tumor nor in the earlier generations of the strains leading up to sarcoma development were any stroma elements with unusual characters seen. However, they observed a definite stage before the appearance of the sarcoma, in which the stroma of the tumor had become more abundant and cel-

lular, and this was followed in later transplants by the emergence of sarcoma. It is possible that transplantation of the pulmonary growths into the subcutaneous tissue may in some fashion stimulate the stroma of the host to malignant transformation. If this is actually the mechanism of sarcoma development in the present experiment, it is not to be explained on the basis of transfer of a known carcinogenic hydrocarbon in the primary tumor, for this phenomenon was seen following the transplantation of tumor 11, which was a spontaneous growth. Furthermore, the spectroscopic studies of Lorenz (*34*) have shown that the carcinogenic hydrocarbons disappear from the lungs of mice within 7 days following injection. Histologic studies of this material, while giving evidence of an active stromal reaction about the periphery of many of the transplanted tumors, have not yielded satisfactory indications that this stromal reaction on the part of the host becomes neoplastic. This possibility is not ruled out because we have not as yet examined sufficient serial material between the time of first transplantation and the eventual appearance of sarcoma.

A third possibility is that the epithelial tumor cells undergo such fundamental alterations in their character as to become transformed into tumor fibroblasts. This possibility has been examined thoroughly in these tumors, and while, as we have previously noted, there sometimes was observed a close physical association between elongated or spindle-shaped epithelial tumor cells and definitely sarcomatous areas of growth, we are unable to conclude that this relationship is any more than incidental.

A fourth possibility that requires further exploration is that the primary pulmonary tumors are in reality mixed tumors to begin with. That they may possibly arise from two or more cell types, viz, alveolar epithelium, mesenchymal alveolar cells, and/or pleural endothelium must be considered. There is much to recommend this line of speculation although the absence of direct proof precludes any positive deductions at present. The so-called endothelial tumors in man, particularly the malignant synovioma, duplicate histologically the carcinosarcomatous features of the primary pulmonary tumor as they develop during successive periods of transplantation. In the synovioma, there are areas which if taken alone would be called adenocarcinoma, other areas carcinoma without acinous structure, and still other areas fibrosarcoma. Indeed, all these errors in diagnosis have been made in the pathologic examination of synovioma. Another parallel between our observations of the transplanted pulmonary tumor and malignant synovioma is that in some areas the epithelial component of the growth shows a glandular arrangement and yet the supporting stroma is obviously malignant and has the appearance of fibrosarcoma. Plans are under way to investigate by precise cytologic methods whether the pulmonary tumor is a mixed tumor from the start. Since the tumors employed in this study were selected from the peripheral surface of the lung and almost certainly contained serosal cells from the pleura, it would be interesting to repeat this work using only deeply situated pulmonary tumors not in contact with the pleura.

Foulds (*35*, *36*) transplanted a carcinoma which he believed had originated in the oviduct of a fowl. A fibrosarcoma arose at the site of implantation of the carcinoma tissue, and he thought that this had developed after the original transplant had regressed. Bone and cartilage were frequently present in subsequent transplants, and the tumors then had a mixed structure. He concluded that the bone was formed by the host's connective tissue cells, which were constrained to differentiate in this way by conditions brought about by the epithelial parenchyma. The bone observed in our tumors was always confined to the stroma of the epithelial growths and was never malignant in appearance.

SUMMARY

Histopathologic studies of 22 primary pulmonary tumors arising spontaneously or induced with 1,2,5,6-dibenzanthracene in inbred mice of several strains showed that during repeated transplantations 11 of these developed a change in the character of the growths, which represents a transition from an epithelial pattern to a fibrosarcoma.

The transplants of the various tumor strains could be divided into several histologic types, which were often intermingled or predominantly of a single type.

The acinous carcinoma type preserved the original glandular character and was uniform in appearance.

The nonacinous carcinoma type possessed an epithelial pattern but did not reproduce acinous formation; instead the epithelial-appearing cells were arranged in columns, nests, or solid sheets.

The sarcoma type possessed all the structural features upon which a histologic diagnosis of fibrosarcoma is based. The various combinations of the types observed are described.

A study of the final histologic status of the various tumors and their collateral lines shows that in 5 lines the tumor transplant had become wholly sarcomatous in type while in an additional 13 lines the tumor was composed of a combination of sarcomatous and carcinomatous structure.

The theoretical mechanisms and underlying causes of sarcomatous transformation following serial transplantation of primary pulmonary tumors of the mouse were explored, but no positive conclusion was reached.

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TUMORS OF THE ALIMENTARY TRACT INDUCED IN MICE BY FEEDING OLIVE-OIL EMULSIONS CONTAINING CARCINOGENIC HYDROCARBONS [1] [2]

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INTRODUCTION

The induction of adenocarcinoma of the small intestine in strain A mice by the oral administration of 1,2,5,6-dibenzanthracene [3] and in strain A backcross mice by 20-methylcholanthrene [3] was previously reported (*1*). The carcinogens were administered in aqueous olive-oil emulsions which were given instead of drinking water. During 1940 new experiments were designed in which olive-oil emulsions containing either methylcholanthrene or dibenzanthracene were administered to mice of six different strains and to A backcross mice. This investigation was undertaken to study the susceptibility of mice of different strains to the induction of tumors of the gastrointestinal tract and to gain additional knowledge as to the factors governing tumor induction by the oral administration of carcinogenic hydrocarbons. The experiments were finished in 1941.

MATERIALS AND METHODS

The hydrocarbons were administered in an aqueous olive-oil emulsion containing 10 percent of olive oil and 0.4 mg. of methylcholanthrene or 0.2 mg. of dibenzanthracene per cubic centimeter, respectively. The minimum melting points of the hydrocarbons were 179.5° C. (corrected) for methylcholanthrene and 267.0° (corrected) for dibenzanthracene. Previously (*1*), it had been shown that an emulsion containing 0.4 mg. of dibenzanthracene per cubic centimeter was toxic for mice of strains A and A backcross. On the other hand methylcholanthrene in the same concentration was less toxic for strain A backcross mice. On the assumption that this would hold true for other strains of mice, the dibenzanthracene emulsion in the present experiment was made with a concentration of 0.2 mg. per cubic centimeter. The method of preparation of the emulsions has already been described (*1*). These emulsions break rapidly when acidified but are stable at a pH above 8.5 and were therefore used at a pH between 9 and 10. However, they can be made stable in acid, by the addition of 0.5 percent of the dioctyl ester of sodium sulfosuccinic acid (Aerosol OT).[4] Breaking of the emulsions is then prevented even at the pH of the gastric juice. The action of the aerosol probably involves attachment of its molecules to the soap film surrounding the oil droplets of the emulsions, thus preventing the neutralization of this film. All experimental groups of mice were given the nonstabilized emulsions, with the exception of two that received the stabilized emulsions. The nonstabilized emulsions were administered in 100-cc., wide-mouth bottles, closed by a rubber stopper, into which was inserted a glass tube 8 mm. wide and approximately 100 mm. long with an opening of a few millimeters at the tip. These bottles were placed perpendicularly through the top of the wooden box. Because of the high wetting power of the aerosol, which caused considerable loss by spilling when shaken even slightly, the stabilized emulsions were administered differently. The animals which received the stabilized emulsions were kept in metal cages with screened top, back, and door. A flat, narrow-mouthed bottle of 100-cc. capacity was fastened rigidly to the door of the cage. The bottle was closed with a rubber stopper into which was inserted a slightly bent glass tube, 8 mm. in diameter and 3 to 4 cm. in length so that only the tip of the tube protruded into the cage. The tip had an opening approximately 1 mm. in diameter. Usually 10 animals were kept in each cage.

Records were kept of the weights of all animals and of the quantity of emulsion consumed. No curves of weight or of the intake of the emulsions are given here since typical curves have already been published (*1*). The average daily intake of emulsion and hydrocarbon per mouse was calculated from the total intake of the group. Intake, however, fluctuated considerably from animal to animal, and often towards the end of the experiment the intake rose by as much as 50 percent for the last surviving animals. The experimental data are presented in tables 1 and 2.

By weighing the experimental animals, especially in the later stages of the experiment and by abdominal palpation and observation for intestinal hemorrhage and signs of obstruction, intraabdominal tumors were frequently diagnosed. Animals which were dying or in which the presence of tumors was suspected, were killed with ether, and the tissues, uncomplicated by post-mortem autolysis, were studied. Only a relatively small number of animals died during the experiment. The pathologic techniques employed have been described elsewhere (*2*).

Control animals of the same age were kept for each group. Animals of one group received the olive-oil emulsion without the hydrocarbon; those

[1] Received for publication January 13, 1947.
[2] Presented at the meeting of the American Association for Cancer Research, Chicago, April 1941.
[3] Hereafter referred to as dibenzanthracene and methylcholanthrene, respectively.
[4] Hereafter referred to as aerosol.

of another group received water. In the experiments in which stabilized olive-oil emulsions were given, a third group received a 0.5-percent solution of aerosol in water. Previous experiments had shown that the addition of 0.1 percent of aerosol to the drinking water ingested by strain A backcross mice for 7 months produced no consistent or significant gross or microscopic lesions (*3*). Fluid intake and weight records were also obtained for all control animals, which were usually autopsied after death of the last surviving experimental animal of that particular group.

TABLE 1.—*Consumption of methylcholanthrene-olive oil emulsions by mice*

Strain	Number of mice	Age at beginning of experiment	Sex	Duration of experiment	Emulsion [1]	Quantity ingested by any single mouse: Average total of— Emulsion per day	Average hydrocarbon per day	Maximum hydrocarbon
		Months		*Days*		*Cubic centimeters*	*Milligrams*	*Milligrams*
C57 brown	12	3½	M	140	Olive oil (10 percent)	3.3	1.3	138
	12	3½	F	140		3.0	1.2	130
C57 black	11	4	M	147		2.8	1.1	121
	12	4	M	171		2.6	1.0	140
C3H	10	3½	M	155		2.3	.9	121
	10	3½	M	156		2.1	.8	102
I	10	2	M	76		1.8	.7	41
	10	2¾	M	90		1.9	.8	51
dba	10	2-3	M	107		1.9	.8	72
	10	2-3	F	122		2.1	.8	83
A	10	3	M	247		1.9	.8	144
	10	3	M	242		1.9	.8	161
	10	3	F	267		1.8	.7	150
A backcross [2]	10	4	M	306	Olive oil (20 percent)	2.2	.9	275
Do	10	3½	M	273	Stabilized olive oil (10 percent)	1.8	.7	182
	10	3½	F	288		1.8	.7	182

[1] 0.4 mg. of methylcholanthrene per cubic centimeter of emulsion.
[2] Data taken from previous experiments (*1*) for comparison.

TABLE 2.—*Consumption of dibenzanthracene-olive oil emulsions by mice*

Strain	Number of mice	Age at beginning of experiment	Sex	Duration of experiment	Emulsion [1]	Quantity ingested by any single mouse: Average total of— Emulsion per day	Average hydrocarbon per day	Maximum hydrocarbon
		Months		*Days*		*Cubic centimeters*	*Milligrams*	*Milligrams*
C57 black	10	4	M	179	Olive oil (10 percent)	3.4	0.7	105
	10	4	M	156		3.6	.7	96
C3H	10	3½	M	297		3.1	.6	175
	10	3½	M	266		3.5	.7	175
dba	10	3-4	M	190		2.8	.6	106
	10	3-4	F	204		2.4	.5	96
A	10	4	M	244	Olive oil (20 percent)[2]	2.0	.8	160
	10	4	M	303	do	1.6	.6	156
	10	4	F	275	do	2.1	.8	192
A backcross [3]	10	1½	M	394	Olive oil (10 percent)	2.5	.5	182
Do	10	3½	M	420	Stabilized olive oil (10 percent)	3.0	.6	242
	10	3½	F	420		3.0	.6	245

[1] 0.2 mg. of dibenzanthracene per cubic centimeter of emulsion.
[2] 0.4 mg. dibenzanthracene per cubic centimeter.
[3] Data taken from previous experiments (*1*) for comparison.

All animals were purchased from the Roscoe B. Jackson Memorial Laboratory at Bar Harbor, Maine, with the exception of the strain I and dilute brown animals which were obtained from the stock of this Institute. All animals were fed Purina dog chow ad libitum which, according to the manufacturer, contained at that time 20 percent protein, 3 percent fat, 56 percent carbohydrate, 6 percent ash, 15 percent water, together with vitamins A and G.

Contrary to expectation based upon the earlier work (*1*), the methylcholanthrene emulsion proved

to be toxic for all strains of mice. In the present experiment, however, it was least toxic for the hybrid A backcross mice. This toxicity was frequently manifested by rapid loss of weight or anasarca. Only by the substitution of limited amounts of drinking water for the emulsion from time to time could death be prevented. The emulsion was especially toxic for the animals of strains I and dba. The mice of these strains survived for periods ranging from 76 to 122 days, corresponding to a maximum intake of hydrocarbon from 41 to 85 mg. This intake is considerably lower than that of the other strains. No deaths from tumor of the gastrointestinal tract occurred in these animals. For strain C57 brown, C57 black, C3H, A, and A backcross mice, the toxicity varied with the daily intake of hydrocarbon ranging from 1.3 mg. for male C57 brown mice to 0.7 mg. for male and female strain A backcross mice. The survival time ranging from 140 to 288 days in these strains is, however, conditioned in part by the development of tumors of the gastrointestinal tract. As stated, the toxic effects of the methylcholanthrene emulsion were not forseen when the present experiment was planned. A reduction of the methylcholanthrene content of the emulsion from 0.4 to 0.2 mg. per cubic centimeter would probably have lessened the toxicity, as the data on consumption of the dibenzanthracene emulsion show. The dibenzanthacene content of the emulsion was reduced to 0.2 mg. per cubic centimeter in contrast with 0.4 mg. per cubic centimeter of the previous experiment in which this emulsion proved to be highly toxic. The emulsion containing the 0.2 mg. of dibenzanthracene per cubic centimeter was of low toxicity for all strains with the exception of strain C57 black; only very rarely was it necessary to substitute water for the emulsion on account of anasarca or rapid loss of weight. This belief is strengthened by the evidence that the dba strain animals on dibenzanthracene emulsion of low content survived almost twice as long as did those given methylcholanthrene emulsion containing 0.4 mg. of that hydrocarbon per cubic centimeter.

The control animals, which were given plain olive-oil emulsion or a solution of aerosol in water, showed no toxic manifestations.

RESULTS

The lesions induced in mice by oral administration of dibenzanthracene and methylcholanthrene olive-oil emulsions have been described previously (*1*). Here data on the type, location, and incidence of, and amount of hydrocarbon necessary to induce lesions in the different strains of mice are presented, together with a more detailed description of any changes not previously recorded.

The lesions involving the mucous membrane of the gastrointestinal tract consisted of (1) carcinoma and precancerous lesions of the small intestine and (2) squamous-cell carcinoma, hyperkeratosis, hyperplasia, papilloma, and dyskeratosis of the forestomach.

Lesions occurring elsewhere consisted of (1) malignant hemangio-endothelioma of mesentery and peritoneum, (2) fibrosarcoma of mesentery, (3) pulmonary tumor, and (4) miscellaneous lesions.

Table 3 shows that single or multiple carcinomas of the small intestine were observed in 75 of 287 mice, or in 26 percent of the animals. Single or multiple precancerous lesions of the small intestine were observed in most of these 75 mice, which also showed intestinal carcinoma, and in 46 additional mice. Carcinoma and the precancerous lesion were located at various points over the entire length of the small intestine, the highest frequency being in a region between 7.5 and 17.5 cm. distal to the pylorus. Figure 1 is a histogram of

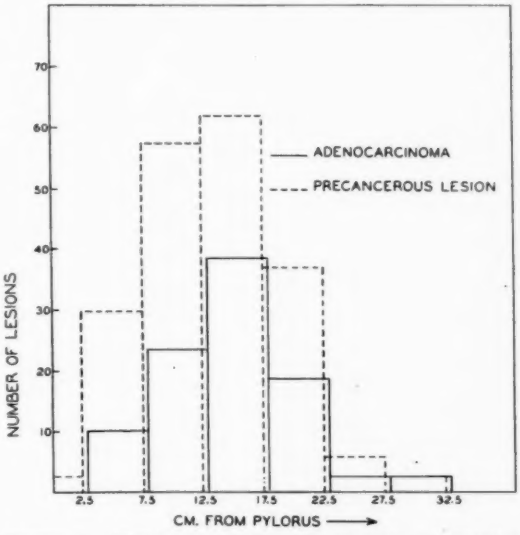

FIGURE 1.—Histogram of the distribution of intestinal carcinoma and precancerous lesions.

the distribution of carcinoma and precancerous lesions. While the number of carcinomas plotted is exact, there may be some question about the accuracy of the incidence of the precancerous lesion since the data were obtained on gross observations at autopsy, and some of the smaller, less conspicuous, early lesions may have been missed. All the precancerous lesions, however, were confirmed microscopically.

The gross and microscopic characteristics of the induced precancerous lesion and carcinoma of the small intestine have been described in other publications (*1*, *2*). For purposes of classification, the arbitrary view has been adopted that a given cytologically atypical, infiltrating, epithelial proliferation of the mucosa of the small intestine is in a precancerous stage until after neoplastic infiltration of all coats of the intestinal wall has occurred and there is a tumor nodule on the underlying peritoneum. Only then is the lesion classified as carcinoma.

Metastases from the intestinal carcinoma were observed at the base of the mesentery and in the mesenteric lymph nodes in three mice. The low incidence of metastasis may be accounted for in part by the method of procedure in this experiment. As soon as an abdominal mass was palpable or the mouse developed tarry stools, anasarca, or signs of intestinal obstruction, or showed any evidence whatever of impending death, it was killed and autopsied. Moreover, the animals were not taken off the established regime of the hydrocarbon emulsions, which added a toxic factor in mice already subject to the ill effects of intestinal tumor. The combination of these factors tended to shorten the life of the animal after the tumor had developed, thereby decreasing the probability of metastasis.

The data on the induction of carcinoma of the small intestine are compiled in table 3. With the exception of mice of strain I, these tumors were observed in all strains of mice. There appears to be a difference in susceptibility between males and females, more males developing tumor than females.

TABLE 3.—*Adenocarcinoma of the small intestine induced with oral administration of methylcholanthrene and dibenzanthracene olive-oil emulsions in mice*

METHYLCHOLANTHRENE [1]

Strain	Number [2]	Sex	Olive-oil content of emulsions	Average total of hydrocarbons ingested until first adenocarcinoma was observed	Duration of experiment until first tumor was observed	Mice with single or multiple adenocarcinoma [3]	Mice with precancerous lesions only [4]
			Percent	*Milligrams*	*Days*	*Number*	*Number*
C57 brown	12	M	10	95	95	6	3
	12	F	10	75	92	5	5
C57 black	11	M	10	95	110	2	2
	12	M	10	89	103	4	2
C3H	10	M	10	86	108	5	3
	10	M	10	82	121	3	2
I	10	M	10	Maximum ingestion of 41 mg. in 76 days		None	None
	10	M	10	Maximum ingestion of 57 mg. in 90 days		None	None
	10	M	10	Maximum ingestion of 72 mg. in 107 days		None	None
dba	10	F	10	Maximum ingestion of 85 mg. in 122 days		None	None
A	10	M	10	121	175	6	2
	10	M	10	99	133	7	1
	10	F	10	117	194	3	4
A backcross [5]	10	M	20	175	227	3	None
Do	10	M	[6] 10	130	197	5	1
	10	F	[6] 10	182	288	2	1

DIBENZANTHRACENE [7]

C57 black	10	M	10	86	150	1	None
	10	M	10	89	156	1	3
C3H	10	M	10	160	270	3	2
	10	M	10	152	239	3	1
dba	10	M	10	108	190	3	6
	10	F	10	81 for first precancerous lesion			5
A [5]	10	M	[8] 20	125	214	1	
	10	M	[8] 20	113	259	5	
	10	F	[8] 20	245	236	1	
A backcross [5]	10	M	[8] 20	Maximum ingestion of 182 mg. in 399 days			
Do	10	M	[6] 10	200	355	4	2
	10	F	[6] 10	245	420	2	1

[1] 0.4 mg. per cubic centimeter of emulsion.
[2] Total number of mice, 287.
[3] Total mice with adenocarcinoma, 75.
[4] Total mice with precancerous lesions only, 46.
[5] Data taken from previous experiment (*1*) for comparison.
[6] Stabilized.
[7] 0.2 mg. per cubic centimeter.
[8] 0.4 mg. of dibenzanthracene per cubic centimeter of emulsion.

The time at which the first tumor was observed in mice of the different strains varied considerably. If, however, the amount of hydrocarbon ingested is calculated up to the time the first tumor was observed, it is found that this figure is similar for strains C57 brown, C57 black, C3H, and A, which were given the methylcholanthrene emulsion. This amount ranges from 75 to 121 mg. Similarly, the amount of dibenzanthracene ingested by strains C3H, dba, and A (strain 57 black excepted) until the first tumor was observed ranges from 108 to 160 mg. Variations in the amount of hydrocarbon ingested during the latent period can be explained in part on the basis that tumors may

have existed for varying lengths of time before they were suspected clinically. Hence, it seems justifiable to assume that susceptibility to intestinal tumor induction of the inbred strains used in these experiments is approximately the same. On the other hand hybrid strain A backcross mice show a considerably longer latent period to the induction of carcinoma of the small intestine since the ingestion of 130 to 182 mg. of methylcholanthrene and of 200 to 245 mg. of dibenzanthracene was required to induce the first tumor.

Precancerous lesions were usually multiple and were observed either alone or in association with carcinoma. As would be expected, they developed before the first carcinoma appeared. The first precancerous lesion was observed after 66 days in a strain C57 brown female mouse, while the first carcinoma was found after 92 days of ingestion of the methycholanthrene emulsion in the same animals. The precancerous lesions are described in the following paper (*2*). They were not observed in the first experiment (*1*); this may be accounted for by the fact that the method of studying the mucosal surface of the entire length of the gastrointestinal tract under a hand lens at autopsy was not then employed, and it was not until later, after some experience in the detection of these lesions had been gained, that we were able to identify them more precisely.

Squamous-cell carcinoma of the forestomach occurred in 14 of 287 experimental mice; there were 4 of 30 strain A mice ingesting the nonstabilized emulsion containing methylcholanthrene and 10 of 20 strain A backcross mice ingesting the stabilized olive-oil emulsion. In 75 additional mice of the total series of 287, one or more of the other forestomach lesions occurred, which include mucosal hyperkeratosis, hyperplasia, dyskeratosis, and papilloma (table 4). The same criteria which were employed to distinguish between precancerous lesions and carcinoma of the small intestine were applied in the case of the forestomach lesions. A diagnosis of squamous-cell carcinoma was not made until after all the coats of the forestomach had been completely infiltrated, and there was a neoplastic nodule on the peritoneum. Experimental data and the pathologic features including incidence of metastasis will be presented in other papers.[5]

The glandular mucosa of the pyloric chamber of the stomach proved to be refractory to the induction of tumor by the oral administration of the carcinogenic hydrocarbons so that no carcinoma or precancerous lesion of the glandular stomach was observed. This is in accord with our previous failure to induce gastric adenocarcinoma by orally administered carcinogens (*1, 4*) and contrasts with induction of this lesion in mice by local injection of methylcholanthrene into the wall of the stomach (*5*).

Malignant hemangio-endothelioma of the mesentery or peritoneum occurred in eight mice of strains C3H and dba and A backcross hybrids (fig. 2, *A* and *B*, and table 4). In an undetermined number of additional mice, minute lesions which were considered possibly to represent the early stage of this tumor were observed. This will be the subject of a separate communication. In another experiment (*6*) in which methylcholanthrene was administered orally, malignant hemangio-endothelioma of the mesentery and peritoneum was also induced, and the gross and microscopic features of this lesion were described. Since this tumor was similar in morphologic characteristics and location in both experiments, no further description is given in this paper.

Fibrosarcoma of the mesentery occurred in 15 mice of the C3H and A strains and A backcross hybrids (fig. 3). This tumor was induced only by methylcholanthrene (table 4). It appeared as a firm gray or white nodule of varying size, the largest single tumor measuring 1.5 x 1.0 x 0.5 cm. Microscopically the tumor was composed of interlacing bundles of spindle-shaped cells which varied moderately in size, shape, and staining, and which showed numerous mitotic figures. Fibroglia fibrils were demonstrated with the phosphotungstic acid-hematoxylin stain. In the silver preparations there was an abundance of recticulum fibers which intimately embraced the cells singly and in small groups. The tumor was vascular and the blood vessels consisted of large, thin-walled, immature, sinusoidal channels.

Multiple primary pulmonary tumors were induced in all groups of the mice of this experiment, with the exception of strain I (table 4). As many as 100 nodules were counted in some animals of strain A and A backcross hybrids. There were fewer nodules in strains of low susceptibility such as C57 brown, C57 black, C3H, and dba. Early neoplastic changes developed in the pulmonary alveoli, resembling those described by Grady and Stewart (*7*). With the exception noted, the tumors were histologically similar to the common primary pulmonary tumor of the mouse.

A few mice of the dba strain, in which the common pulmonary tumor just described occurred, also showed another type of pulmonary lesion which has not been encountered in the lungs of any of the numerous other mice studied in this Institute. This lesion consisted of an aggregation of distinctly outlined acinar structures lined by a single layer of cuboidal or columnar cells (figs. 4 and 5). The nuclei were hyperchromatic and basally situated. Between the nucleus and the free border of the cell the cytoplasm was usually

[5] In preparation.

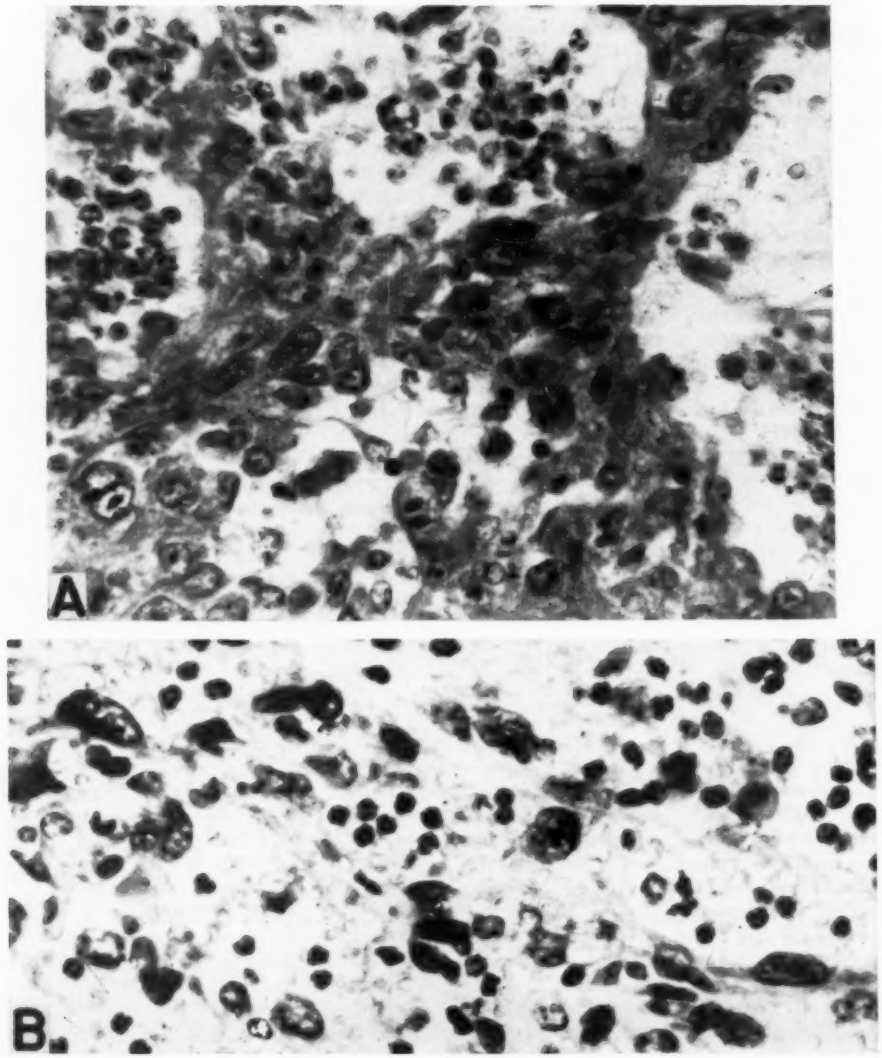

FIGURE 2.—Hemangio-endothelioma of mesentery showing vascular spaces. *A*, Vascular spaces lined by stratified, malignant, endothelial cells. × 320. *B*, Vascular spaces lined by a single layer of endothelial cells, the nuclei of which are enlarged and bulge beadlike into the lumina of the blood-containing spaces. × 415.

TABLE 4.—*Other tumors and lesions induced with methylcholanthrene and dibenzanthracene*

Strain	Number	Sex	Olive-oil content of emulsions	Mice with squamous-cell carcinomas of forestomach	Mice with hyperkeratosis and papillomatosis of forestomach	Mice with fibrosarcoma of mesentery	Mice with hemangioendothelioma of mesentery	Mice with pulmonary tumor
			Percent	*Number*	*Number*	*Number*	*Number*	*Number*
METHYLCHOLANTHRENE [1]								
C57 brown	12	M	10					1
	12	F	10		2			1
C57 black	11	M	10		5			2
	12	M	10		1			
C3H	10	M	10		2	3		1
	10	M	10		2	5		
I	10	M	10		5			
	10	M	10		4			1
dba	10	M	10		1			1
	10	F	10		3			3
A	10	M	10	1	7	3		10
	10	M	10	1	6	2		10
	10	F	10	2	7			10
A backcross [2]	10	M	20					7
Do	10	M	[3] 10	6	3	2		9
	10	F	[3] 10	4	6		1	10
DIBENZANTHRACENE [4]								
C57 black	10	M	10					
	10	M	10		2			
C3H	10	M	10					8
	10	M	10		1		1	7
dba	10	M	10				4	8
	10	F	10		3		2	7
A	10	M	20					8
A [2]	10	M	10					8
	10	F	10					6
A backcross [2]	10	M	10					7
Do	10	M	[3] 10		7		1	10
	10	F	[3] 10		8			8

[1] 0.4 mg. per cubic centimer of emulsion.
[2] Data taken from previous experiment (*I*) for comparison.
[3] Stabilized.
[4] 0.2 mg. per cubic centimeter of emulsion.

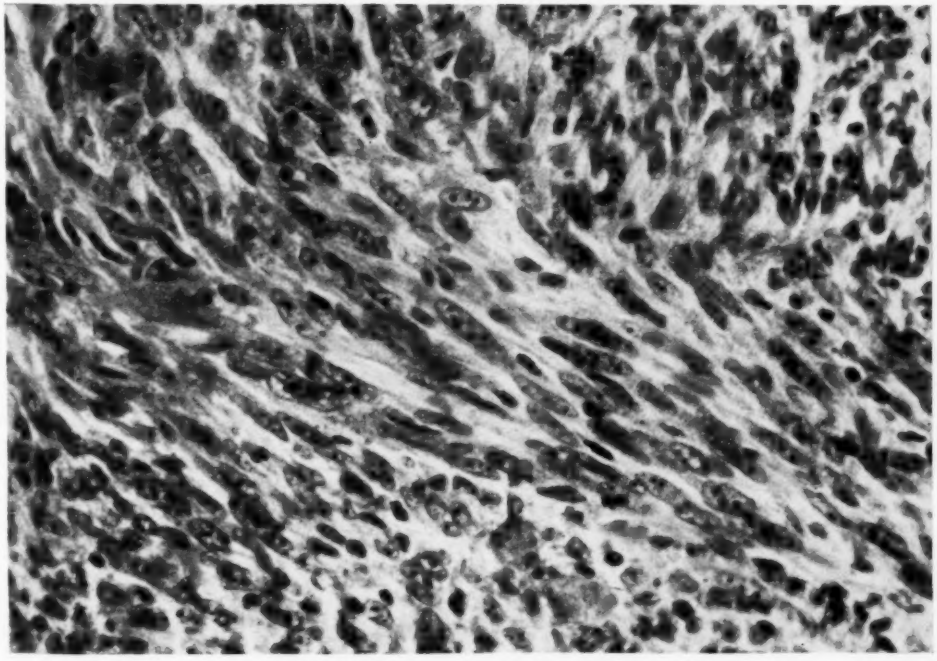

FIGURE 3.—Fibrosarcoma of mesentery. × 285.

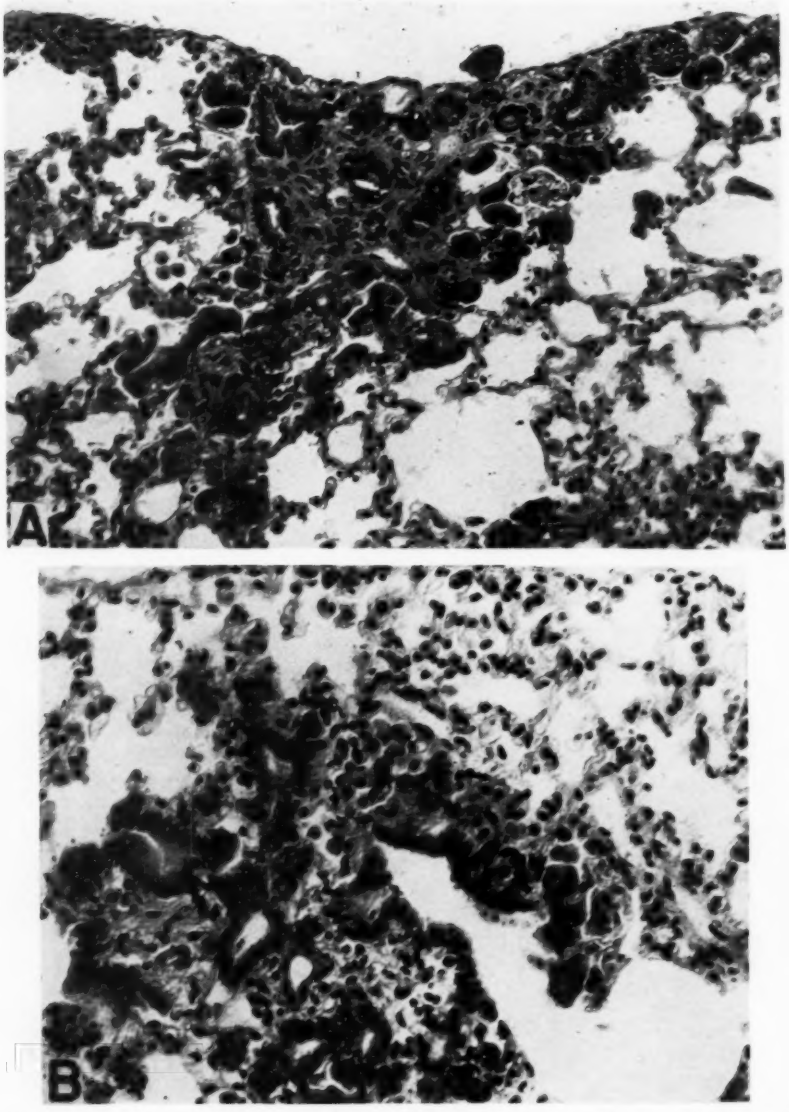

FIGURE 4.—Adenomatous lesion of the lung. *A*, Lesion subplural in position showing glandular structures within a matrix of dense connective tissue. The pleural surface overlying the lesion is slightly depressed. × 195. *B*, Lesion situated more deeply within the pulmonary tissue. × 225.

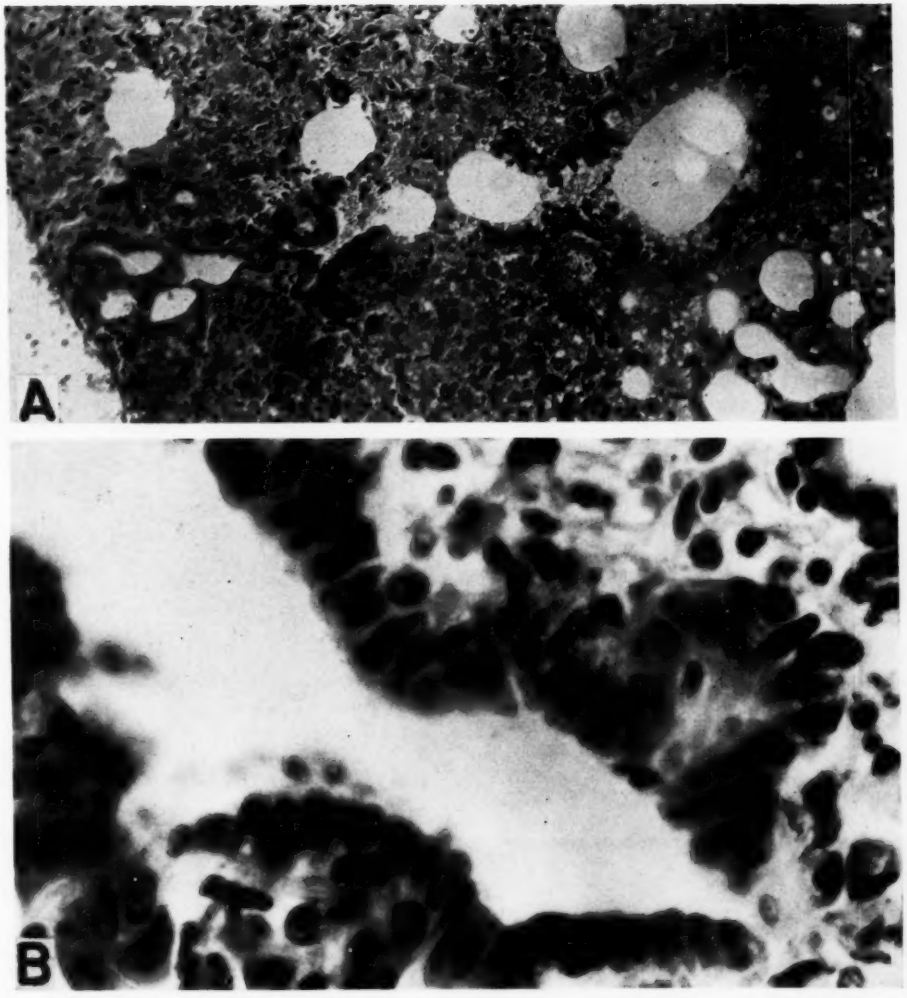

FIGURE 5.—*A*, Adenomatous lesion of lung in close proximity to a bronchiole suggesting that the two may be continuous. × 155. *B*, High-power view of portion of lesion shown in figure 4, *B*, illustrating the appearance of the tall epithelium. × 475.

clear suggesting the presence of some secretory material. Within the area of involvement, the atypical acini were usually separated from each other by a few pulmonary alveoli which were partially collapsed, showed fibrous thickening of their walls, and contained a few macrophages. A bronchiole or large alveolar duct was frequently but not invariably associated with the apical, more deeply placed portion of this lesion. There was a striking resemblance between the epithelium of the acinous structures and that of the bronchi. Some of the lesions were well within the substance of the lung but the majority were superficial. When subpleural in position the surface of the lung was slightly depressed in an area overlying the nodule. Before deciding with regard to the neoplastic or nonneoplastic character of this lesion, further studies will have to be carried out, but it is our impression that this lesion may be bronchial dilatation and proliferation in association with inflammation, rather than a neoplasm. Its possible relationship to pulmonary adenomatosis in man and to jaagsiekte in sheep is under consideration.

Under the heading of miscellaneous lesions are anasarca, and organ and tissue atrophy involving especially the hematopoietic and genital systems as described in an earlier communication (*1*). The changes were similar in all respects to those observed previously.

Occasional specimens of the liver showed myeloid metaplasia. In a few mice of the dba strain the liver showed leukemic infiltration which coexisted with neoplastic involvement of the lymph nodes. Focal bile-duct proliferation and fibrosis, and solitary, grossly visible, cystic dilatations of bile ducts (fig. 6), together with the presence of ceroid pigmentation of the liver, chiefly confined to the Kupffer cells, occurred in a few mice, most of which were of the dba strain.

The control animals remained healthy throughout the experiment and had normal weight curves. The only lesions observed in these animals were (1) pulmonary tumors in strain A and strain A backcross mice which showed the expected spontaneous incidence of this neoplasm, and (2) slight hyperkeratosis and a few papillomas of the forestomach in a number of mice, especially in those ingesting the plain stabilized olive-oil emulsion or the 0.5-percent aerosol solution.

DISCUSSION

Tumors of the alimentary tract induced in the experimental mice by the feeding of olive-oil emulsions containing methylcholanthrene or dibenzanthracene are restricted to the forestomach and small intestine. What information can be obtained from these data about the factors governing tumor induction by the oral administration of olive-oil emulsions containing hydrocarbons? It may be assumed that to induce a tumor a carcinogenic agent must be present at the site of induction for a certain minimum length of time (latency period) depending upon the concentration of the hydrocarbon and the susceptibility of the tissues, and that intermittent presence of the agent increases the latency period, other factors being equal. If these assumptions are accepted, then the following is offered as an explanation for restriction of the induced tumors to forestomach and small intestine. Tumors of the oral cavity or esophagus were not obtained probably because the time of contact of the carcinogen with these organs was short and intermittent. Although mice drink frequently, they also eat frequently, and, therefore, any carcinogen remaining in the mouth or esophagus would be carried into the stomach by food and saliva. However, if the carcinogen is administered in the food, tumors of the oral cavity

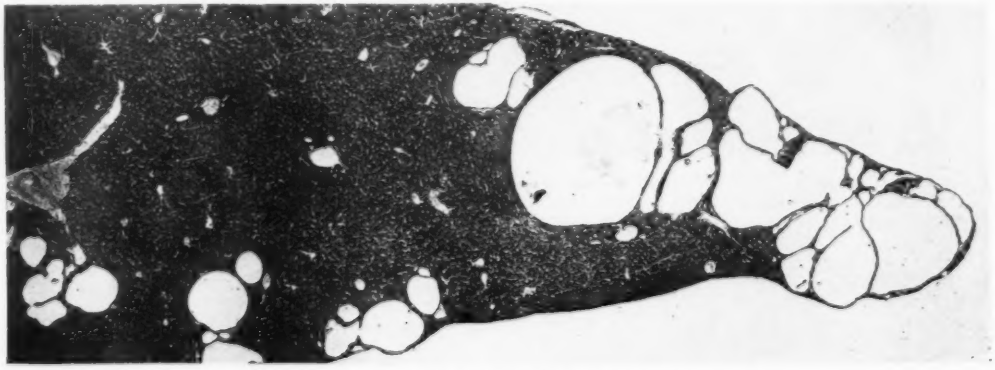

FIGURE 6.—Liver showing cystlike structures. × 33.

are observed (*5*) probably because food particles adhere to the oral cavity thus prolonging the contact of the carcinogen with these tissues.

In the forestomach, storage of food and emulsion favors prolonged contact of the carcinogen with the squamous epithelium. However, because of the instability of the olive-oil emulsion, which breaks and forms oil globules when acidified by the gastric juice, contact of the carcinogen with the wall of the forestomach is poor. Hence only 4 squamous-cell carcinomas were induced with this emulsion. These tumors occurred in strain A mice only, suggesting strain differences in susceptibility. When the olive-oil emulsion was stabilized with aerosol, it withstood acidification to a pH of 1.5 without breaking. Following administration of this emulsion, 10 of 20 strain A backcross mice developed this squamous-cell carcinoma. In contrast, no lesion of the forestomach was found in strain A backcross mice when the nonstabilized emulsion was given. To assume that aerosol is a cocarcinogen introduces unwarranted complexity. It seems more rational to assume that the stabilized emulsion coats the wall of the forestomach, and hence more or less continuous contact between the squamous epithelium is assured especially as the wetting agent also may penetrate the thin protective layer of mucus usually present.

Conditions are different in the glandular stomach. Any coating of the mucosa with the emulsion is probably prevented by continuous secretion of the glands. Moreover, as the food passes relatively rapidly through the glandular stomach, the contact of the carcinogen with the glandular mucosa may be so slight, and therefore the time of latency so long that the animals die of a tumor of the forestomach or of the small intestine long before any tumor of the glandular stomach could develop.

Upon entering the small intestine the olive oil of the nonstabilized emulsion is re-emulsified by the bile while the stabilized emulsion enters the small intestine as emulsion. At the same time digestion of the olive oil begins. Ultraviolet absorption analysis (*1*) has shown that the carcinogen is present in large amounts in the small intestine but that it is not detectable in other tissues and body fluids. This seems to indicate that the carcinogen is precipitated during the digestive process, coming into intimate contact with the glandular mucosa of the small intestine, thus favoring carcinogenesis. The observation of the highest frequency of intestinal carcinoma and precancerous lesions in the region of 7.5 to 17.5 cm. from the pylorus may be explained by assuming that either this region is more susceptible than the others or that in this region the greatest digestion of olive oil and deposition of hydrocarbon take place.

No tumors were induced in the large intestine. The carcinogen was demonstrable down to the level of the ileocecal junction (*1*). In the large intestine no carcinogen was present as such, but a new compound apparently related to it was found. Although the ultraviolet absorption analyses were only carried out for dibenzanthracene, the fact that methylocholanthrene also induced no tumors of the large intestine leads to the assumption that the methylcholanthrene was also converted into a new compound. Whether this conversion of the hydrocarbon is accomplished by the intestinal flora or by secretions from the mucosa of the large intestine has not been determined at present. The conversion product of dibenzanthracene seems not to be carcinogenic. Ether extracts of dried fecal material have been dissolved in lard and injected subcutaneously into strain C3H mice. No tumors were induced.[6]

Although the ultraviolet absorption analysis (*1*) showed no detectable ($<10^{-4}$ mg.) amounts of dibenzanthracene except in the small bowel, even after prolonged administration of the carcinogen, the occurrence of multiple pulmonary tumors and sarcoma and hemangio-endothelioma of the mesentery favor the assumption that small amounts of the carcinogen are absorbed by the small intestine. That we may not have to deal with an indirect (systemic) effect in the induction of the lesions outside the alimentary tract, but with a direct (local) effect of carcinogen absorbed into the general circulation is evidenced by other experiments. When a mineral-oil emulsion, containing the same amount of hydrocarbon as the olive-oil emulsion, is administered orally, the only lesions observed outside the alimentary tract are pulmonary tumors which are present in considerably smaller number than when the olive-oil emulsion is administered. Mineral oil, not being digested in the alimentary tract, probably releases very little carcinogen for absorption into the general circulation. The experiments dealing with the administration of mineral-oil emulsions will be the subject of a subsequent paper.[7]

SUMMARY AND CONCLUSIONS

Six strains of mice (C57 brown, C57 black, C3H, I, dba, A) and A backcross hybrids of both sexes were given orally, aqueous olive-oil emulsions containing either methylcholanthrene or dibenzanthracene in place of drinking water.

[6] LORENZ, E.: Unpublished data.
[7] In preparation.

Some mice of all strains, with the exception of strain I, developed precancerous lesions and carcinoma of the small intestine.

While olive-oil emulsion only rarely induced squamous-cell carcinoma of the forestomach, stabilized olive-oil emulsion readily induced this lesion.

Neoplastic lesions observed outside the gastrointestinal tract included fibrosarcoma and hemangio-endothelioma of the mesentery and pulmonary tumors.

Factors governing tumor induction by oral administration of carcinogenic emulsions are discussed.

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HISTOPATHOLOGY OF INDUCED PRECANCEROUS LESIONS OF THE SMALL INTESTINE OF MICE [1]

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INTRODUCTION

This paper deals with the early morphologic changes which eventuate in carcinoma of the small intestine in mice treated orally with the carcinogenic hydrocarbons 20-methylcholanthrene and 1,2,5,6-dibenzanthracene. Other papers dealing with the effects of the oral adminstration of the carcinogenic hydrocarbons have been published (*1-3*) or are in preparation, hence the present paper is restricted to an account of the induced precancerous lesions of the small intestine. It may be impossible to define at just what point any given precancerous lesion of the intestine begins on the one hand and becomes cancer on the other hand, but we have adopted the arbitrary stand that a lesion is considered definitely malignant only after infiltration of all coats of the intestine has been accomplished and there is a neoplastic nodule on the underlying peritoneum. This study, therefore, embraces all the various changes that antedate this final set of conditions that characterizes the carcinoma. How often any or all of the various combinations of morphologic features described herein precede the frankly malignant intestinal tumor and if and at what stage the lesion is ever reversible are questions which have not been settled and are not considered. A brief preliminary report dealing with the histogenesis of induced experimental carcinoma of the small intestine has been published previously (Stewart and Lorenz (*4*)).

MATERIALS AND METHODS

The materials and methods employed in this study, including the data on the strains of animals and composition of emulsions, were described in detail in previous publications (*1–4a*) dealing with the effects of the oral administration of 1,2,5,6-dibenzanthracene and 20-methylcholanthrene to mice.

A relatively small number of animals died during the course of the experiments. Animals that were dying were killed with ether or by cervical dislocation. Animals were also killed at intervals for pathologic study. The autopsy was performed immediately after death to obviate post-mortem changes. When the animal died, it was not always possible to carry out the autopsy immediately after death, but in the study of the tissue sections, ample allowance was made for the possible complicating factor of post-morten autolysis. At autopsy, the entire intestinal tract was rapidly dissected free and spread out on a wet paper towel. It was usually opened with a pair of dissecting scissors, and the mucous membrane was inspected for lesions. All areas showing suggestive gross lesions as well as representative segments of the intestine from different areas were selected for microscopic study. These specimens were dropped in the fixative while still spread out on the paper. In other cases, the specimens for microscopic study were taken without opening the intestine. Specimens of the organs and tissues were usually fixed in acid Zenker's fluid. Occasionally, other fixatives were used such as formalin-Zenker, dilute formaldehyde solution (U. S. P. 1:10), Tellyesniczky's fluid,[2] and Bouin's fluid. Some tissues were frozen and stained for fat; the rest were embedded in paraffin, cut and stained with hematoxylin and eosin, eosin-methylene blue, hemalum-eosin, phloxine-methylene blue, phosphotungstic acid-hematoxylin, van Gieson's connective tissue stain with and without counterstain. Mallory's connective tissue stain, Foot's silver stain for reticulum, and Verhoeff's elastic tissue stain. Unless otherwise stated, all illustrations are from the small intestines, and the sections are stained with hematoxylin and eosin.

GROSS DESCRIPTION

The gross characteristics of induced experimental carcinoma of the small intestine in mice have been described in other publications (*1, 3-4a*) and are reviewed herein only insofar as they relate to the precancerous lesion (figs. 1–5).

The small intestine of mice receiving the emulsions containing carcinogenic hydrocarbons is usually relaxed, pale, edematous, and smooth, and the coils are more rounded than normally. This altered appearance may extend from the first part of the duodenum to the ileocecal junction but is regularly present in the jejunum. When water is substituted for the emulsions for a week or more immediately preceding autopsy, the small intestine is apt to lose this appearance, hence it can be attributed directly to the effect of the continued administration of the emulsion.

The distribution of the precancerous lesions is exactly like that of the intestinal carcinomas. Although they have been observed widely scattered throughout the duodenum, jejunum, and ileum,

[1] Received for publication December 10, 1946.

[2] Two parts formol, 20 parts 70 percent alcohol, and 1 part acetic acid.

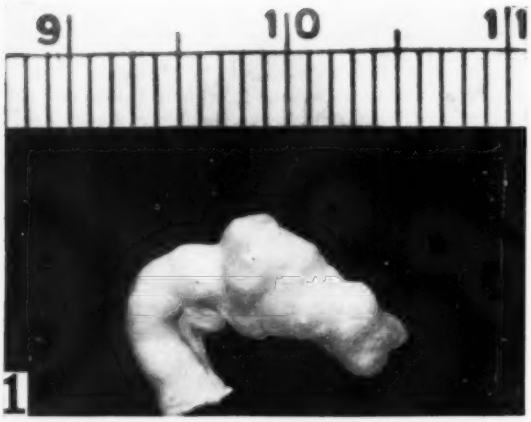


FIGURE 1.—Annular primary carcinoma, gross specimen. × 4.

FIGURE 2.—Primary carcinoma mucosal aspect. Gross specimen. × 8.

most of them are about 15 cm. from the pylorus. When the small intestine is opened, after it has been dissected from the body of the mouse, the earliest, grossly detectable, precancerous lesions, which were first observed in our second experiment, present distinctive features. They are sharply circumscribed, 1 to 4 mm. in diameter, with a central umbilicate depression surrounded by a slightly elevated, smooth, firm, circular border which stands out in sharp contrast against the velvety background of the surrounding mucosa, best identified when viewed under the hand lens (fig. 6). Occasionally tumors develop in the region of the lymphoid follicles. Depending upon the duration of the experiment, the composition of the emulsion, and the strain of the animal, the precancerous lesions may be single or multiple, widely scattered throughout the length and breadth of the small intestine or localized to a confluent patch of multicentric lesions involving a segment 3 to 9 cm. in length, which is thickened and rigid and resembles in miniature the so-called "garden hose lesion" observed in intestinal lymphomas in man (fig. 7).

To distinguish between the precancerous lesion and frank carcinoma is not always possible grossly, since the former merges imperceptibly into the latter. A reliable, but by no means infallible criterion is the diameter of the lesion. Most localized precancerous lesions are not over 4 mm. in diameter. Larger lesions are usually carcinoma. Carcinoma is apt to show more elevation on the mucous surface although almost never does it become polypoid. When carcinoma is present, umbilication is apt to be absent, the lesion being more plaquelike (fig. 2) or frankly ulcerated and there is a variable degree of annular constriction (fig. 1). A palpable nodule on the peritoneum opposite the mucosal lesion is always suggestive of cancer but may be due to an area of organizing granulation tissue. Any of these gross features may fail to be diagnostic, however, and one is sometimes surprised to find microscopically relatively large areas of mucosal thickening or areas of erosion, beneath which the atypical glands are still restricted to the submucosa. The corollary of this is also observed. The final decision whether a given lesion is precancerous or carcinoma, according to the criteria which we have established, must therefore rest on the miscroscopic examination.

The contents of the small intestine are usually liquid when mice are autopsied during the regime of the hydrocarbon-emulsion ingestion. If deprived of the emulsion for a few weeks, the intestinal contents resume their normal appearance and consistency, which is correlated with diminished edema of the intestinal wall. There are sometimes bright red hemorrhages which can frequently be traced to a given precancerous lesion or a carcinoma. Occasionally tarry material is present, indicating old hemorrhage. In this respect, the precancerous lesion and carcinoma in the mouse do not differ from those of man, in whom melena is a frequent diagnostic sign of cancer of the gastrointestinal tract.

MICROSCOPIC OBSERVATIONS

The evolutionary progress of the precancerous lesion as observed in this experiment is illustrated in the photomicrographs. The earliest departure from normal, which has been repeatedly observed, may involve a single intestinal gland or several

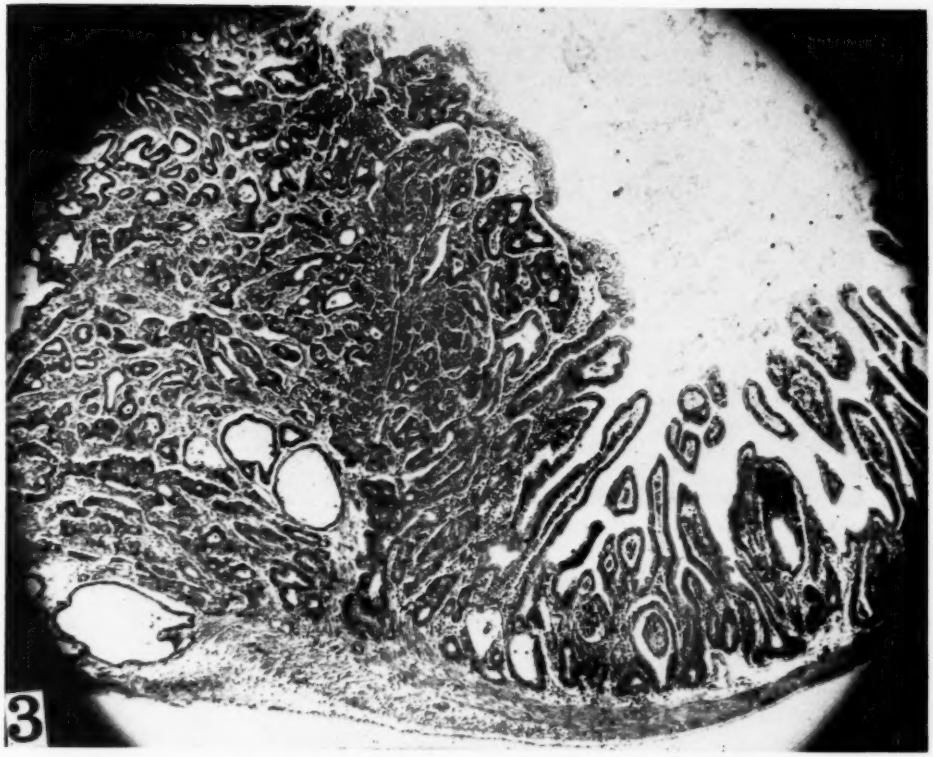


FIGURE 3.—Junction of mucosa with carcinoma. × 56.

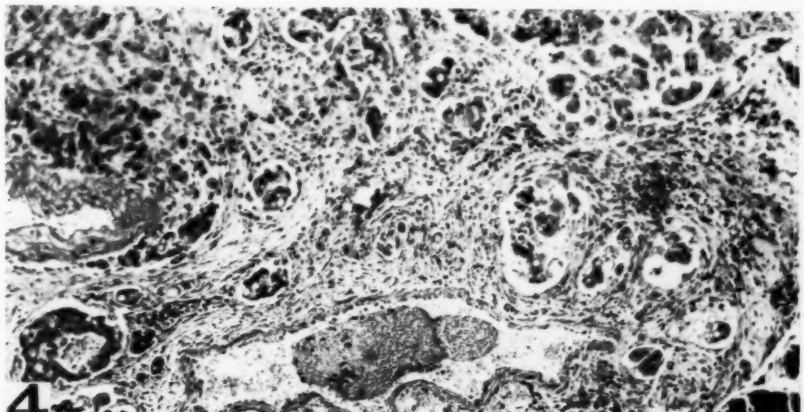


FIGURE 4.—Metastasis at base of mesentery, showing permeation of lymphatic vessels. Portion of pancreas is included. × 105.

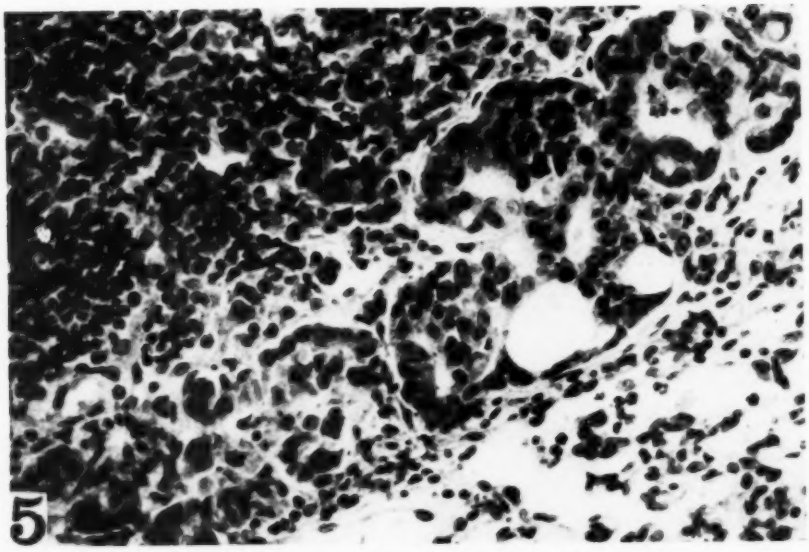


FIGURE 5.—Metastasis in mesenteric lymph node. × 305.

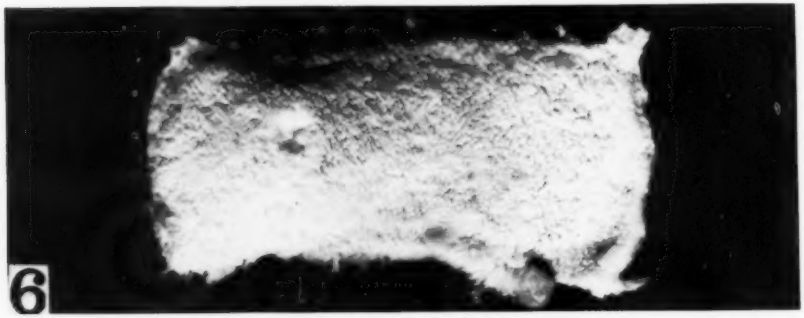


FIGURE 6.—Early precancerous lesion, sharply circumscribed, with a depressed center surrounded by a slightly elevated, smooth margin, which stands out in sharp contrast against the velvety background of the surrounding mucosa. Gross specimen. × 6.

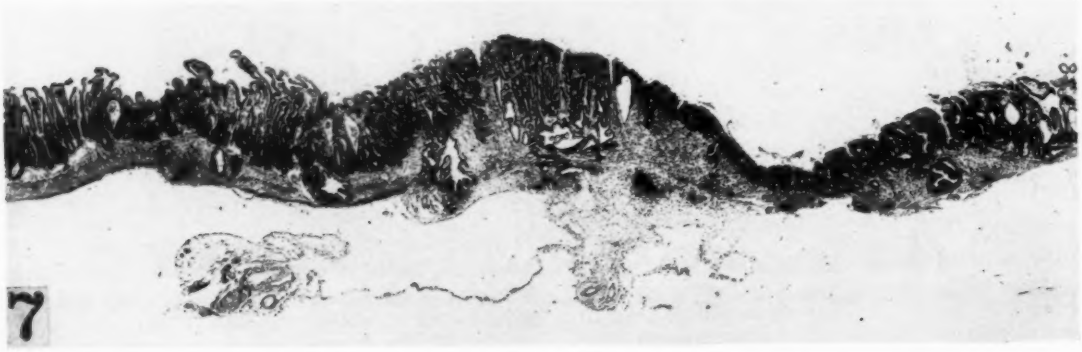


FIGURE 7.—Multicentric precancerous lesions and carcinoma. Circa × 6.

(figs. 8, 9). These foci enlarge and are followed by new foci, which develop independently within the area of involvement. The hyperplastic mucosa reveals crowded glands, in some of which the epithelium is several layers deep, with dark staining nuclei and numerous mitoses (figs. 10, 11, 12). The new formed glands are not only much dilated, longer and wider, but often labyrinthine owing to irregular papillations and complicated branching. Multiple-cell layers are usually found at least in some of the glands, often in association with increased complexity and tortuosity. There is folding due to excessive crowding and solidly arranged cords and nests of cells without acinous formation. Some cells are hypertrophied, others are flattened consisting of undifferentiated cells, mucous cells, and Paneth cells in varying proportion. The heavily stained nuclei may contain 2 or 3 nucleoli and show 5 to 15 mitoses per high-power field unevenly distributed. The cytoplasm tends to be deeply basophilic. In the lumina of the glands there are cellular detritus and structures resembling bacterial and yeast forms.

The acini give rise to small buds, at first solid and later lumenizing, which while still in continuity with the parent gland have a common basement membrane, which is pushed ahead by the growing bud. As these buds enlarge and secondary ones develop, they become branched and complicated and are supported by a new reticulum. Parts of the complicated gland atrophy, or become infected and undergo degeneration and disappear. New buds form; and the old buds continue to enlarge, or they in turn degenerate until finally there is a new generation of acini whose potentialities for growth and infiltration are increased.

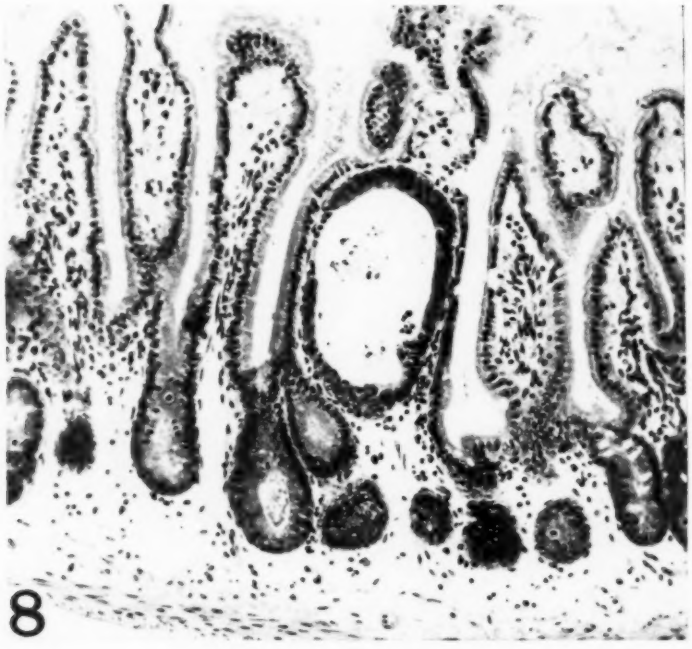
Umbilicated lesions are depressed below the surface and show hyperplastic basal glands and atypical foci in the midmucosa, that is, the portion lying midway between the base of the glands and the mucosal surface. On either side, the border is edematous and elevated by dilated, elongated, atypical glands, and overhanging hyperplastic villi, which blend into the more normal mucosa laterally. The area enlarges by deep infiltration, lateral spread, and the confluence of closely adjacent growths. The surface becomes deeply eroded and ulcerated, is usually covered with mucus and cellular detritus, and often shows gross infection (figs. 13–18).

Occurring either in association with or independently of the umbilicated lesion, the focal and diffuse glandular proliferation at the base of the mucosa results in the downward extension and infiltration of the underlying stroma and muscularis. These epithelial infiltrations begin as downward bulbous or tubular elongations of the crypts of the glands. The continuity between these deeply placed, newly proliferated acini and the overlying original mucosa is ultimately severed. The former assume a progressively complicated neoplastic pattern. The original glands of the superficial mucosa, immediately overlying the site where the basal proliferation is taking place, at first show pronounced swelling and edema, with mucoid droplets within the epithelial cells, dilatation of the lumina, and edema and degeneration of the stroma, followed in time by atrophy, exfoliation, ulceration, and inflammation so that the villi take on a withered appearance and finally disappear. The newly proliferated neoplastic basal structures replace the lost mucosa and are then in contact with the lumen of the intestine (figs. 19, 20, 21).

The so-called "garden hose" type of lesion (fig. 7) consists of a relatively long segment of small intestine measuring 3 to 9 cm. in length and showing all possible combinations of the early and late neoplastic changes. Individual discrete neoplastic foci may be so close together that their separation by narrow wedges of relatively normal mucosa is not appreciated until the histologic preparation is examined. These intervening wedges of relatively normal mucosa become lost as the early neoplastic foci enlarge and coalesce.

The histologic techniques employed in these experiments were not primarily designed for a study of the Paneth cell, the argentaffin cell, and the goblet cell in their possible evolutionary relationship to each other in the developing cancer. The observations on the Paneth cell are open to the least criticism from this point of view since excellent pictures of them have been obtained with Zenker's fixation and subsequent staining with eosin-methylene blue or hemalum-eosin. The goblet cells are usually distinct in these preparations or in those stained with hematoxylin and eosin. Argentaffin cells have often been noted, sometimes in abundance, in the developing carcinoma in sections stained with silver. But again, their precise relationship to the complicated mechanisms underlying the development of induced carcinoma of the intestine must await further detailed controlled study of especially handled histologic material, employing the methods recommended by Popoff (5).

Paneth cells show proliferative activity throughout the entire course of neoplastic development. They are most numerous in the crypts of the elongated, proliferating glands at the base of the mucosa. They vary numerically from area to area. Various acini contain one or two, sometimes a dozen or more Paneth cells, or as many as one-half of the cells are Paneth cells. Large numbers are occasionally seen in areas where the growth is more solid and less acinous. Not infrequently acini containing Paneth cells have their lumina filled with



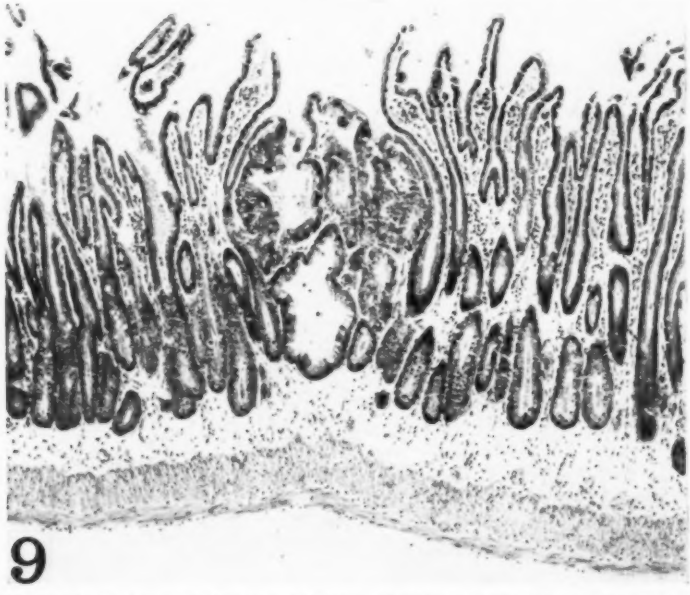


FIGURE 8.—Early precancerous changes. There is a solitary atypical acinus about the mid-portion of the mucosa; basal glands show proliferation and hyperchromasia. × 155.

FIGURE 9.—Small, atypical, glandular focus in the midmucosa and proliferation of the basal glands, below which there is slight retraction or tenting of the muscularis. × 75.

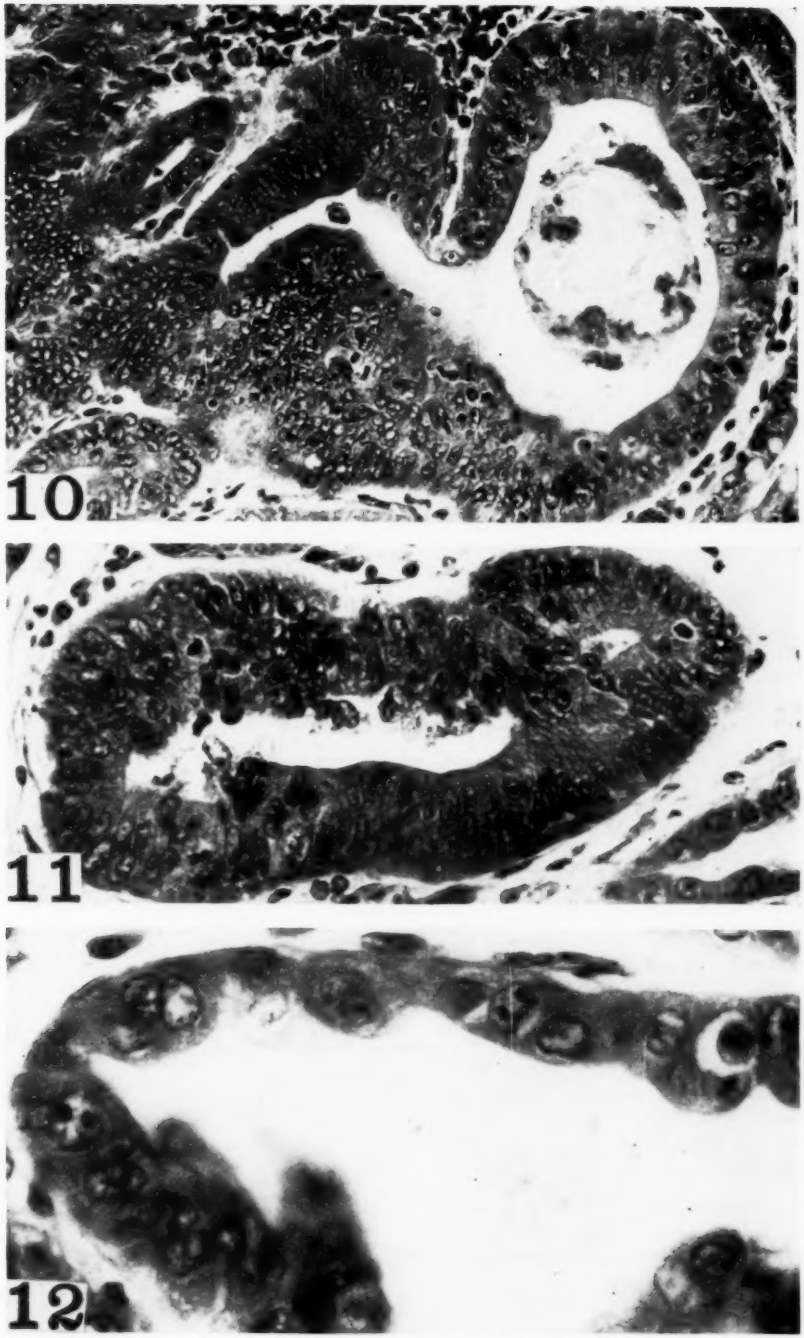
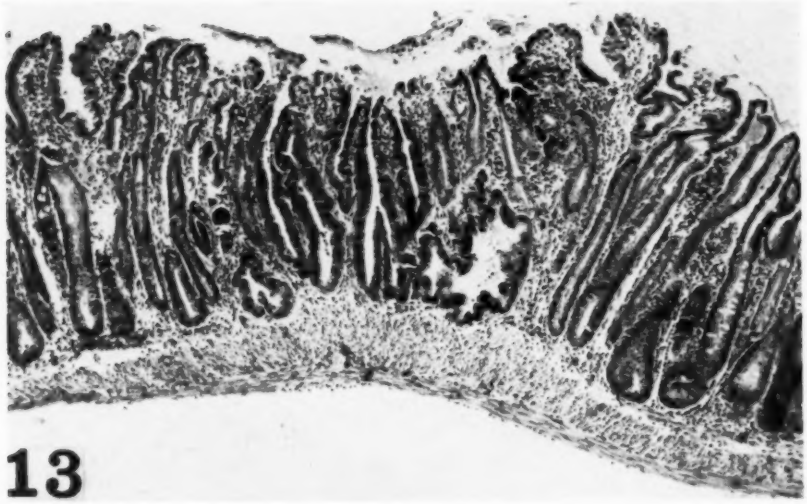


FIGURE 10.—Atypical, complicated mucosal gland from an area showing precancerous changes. × 335.

Figure 11.—Atypical gland in a precancerous lesion. × 480.

FIGURE 12.—Early atypical gland showing change in polarity and variation in size, shape, and staining of cells. × 900.



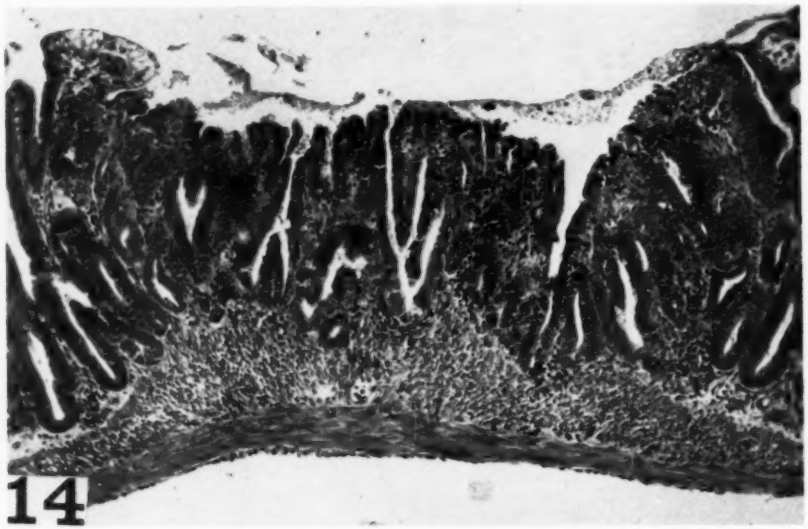
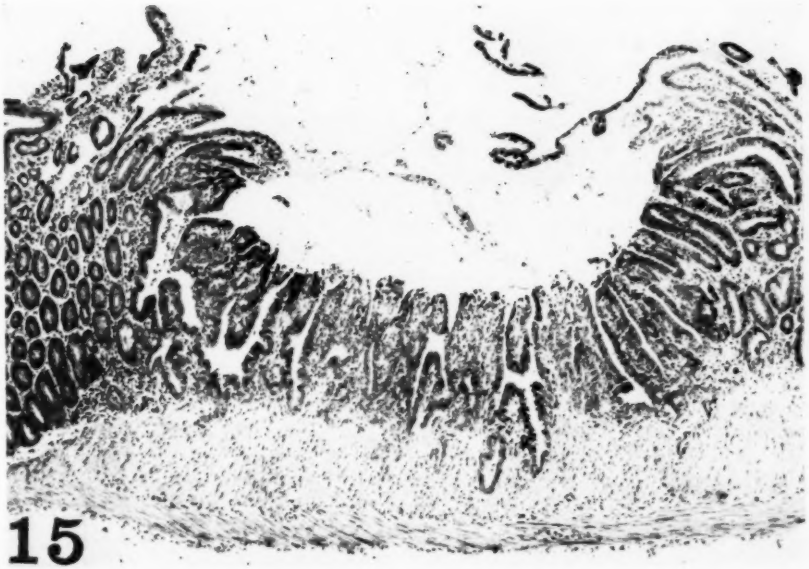


FIGURE 13.—Slightly depressed surface above the area of mucosa, which shows a combination of atrophy and proliferation. Thickening of the inner layer of the muscularis is beginning. × 80.

FIGURE 14.—Another umbilicated lesion showing early infiltration of the thickened circular muscularis. × 112.



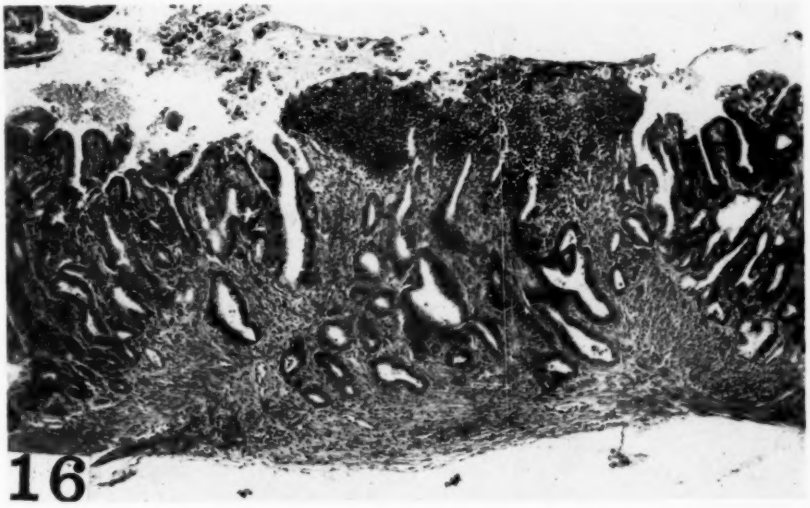


FIGURE 15.—Umbilicated lesion showing the overhanging margins of hyperplastic mucosa. At the site of the umbilicated area the original mucosa has been lost. There is early neoplastic infiltration of the muscularis. × 74.

FIGURE 16.—Umbilicated area showing infection and ulceration of the superficial portion of the neoplasm and in its deeper aspect infiltration through the muscularis. × 76.

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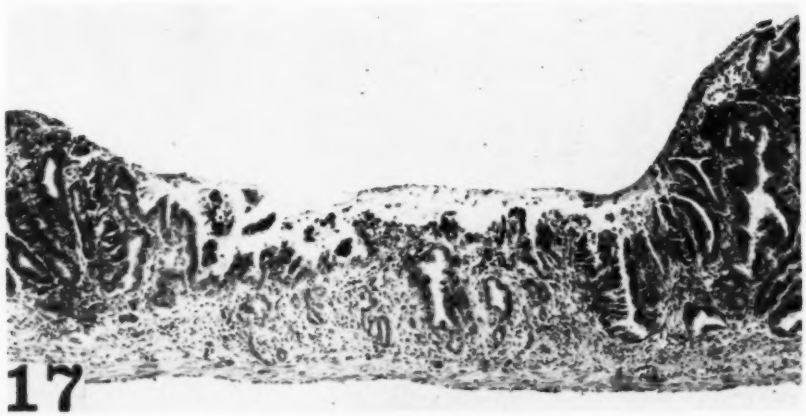


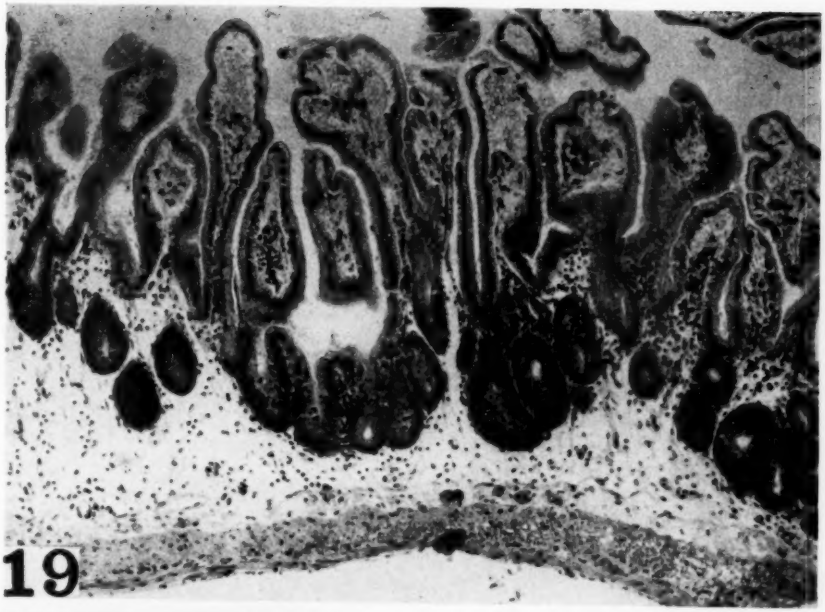
FIGURE 17.—Ulceration extending down to the muscularis, which is infiltrated by gland structure. × 75.

FIGURE 18.—Entire wall of the gut is replaced by infiltrating carcinoma. × 40.

refractile, acidophilic, granular material and degenerated cellular detritus, giving the inescapable impression that these materials have originated from the Paneth cells. The Paneth cells are observed in this material at all levels of the infiltrating neoplasm both in the precancerous stage and in the carcinoma, including the peritoneal and mesenteric deposits and the lymph node metastases. One early carcinoma, which showed recent infiltration through the muscularis, was composed of approximately 85 percent Paneth cells. In another case, a metastasis in a mesenteric lymph node consisted of about equal proportions of Paneth cells, signet-ring cells, and undifferentiated tumor cells. In many of the precancerous lesions, single or multiple foci are composed exclusively or in part of swollen mucous cells infiltrating in cords and nests with and without acinous formation (fig. 22).

No squamous carcinomas or adenoacanthomas of the small intestine have been induced by the methods employed in this work. In one case, there was a squamous-lined cyst at the base of a lymph follicle in the small intestine; in the overlying mucosa there were a few foci of squamous metaplasia. There was no evidence, however, of malignant degeneration in these squamous-cell areas.

The lamina propria and to a less extent the submucosa show pronounced changes during the entire course of development of carcinoma. The lymph vessels are numerous and distended, the capillaries are hyperemic, and the cytologic picture is changed (figs. 23, 24). The normal cellular pattern is replaced by a diffuse and focal inflammatory infiltration in which there are varying percentages of polymorphonuclear and mononuclear leukocytes, plasma cells, and lymphocytes, many showing degeneration. The phagocytes often con-



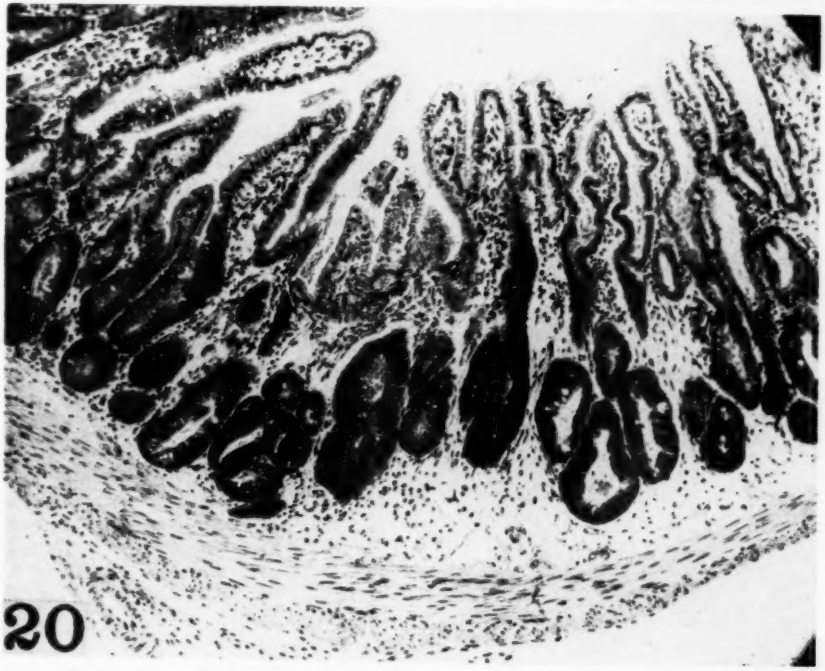
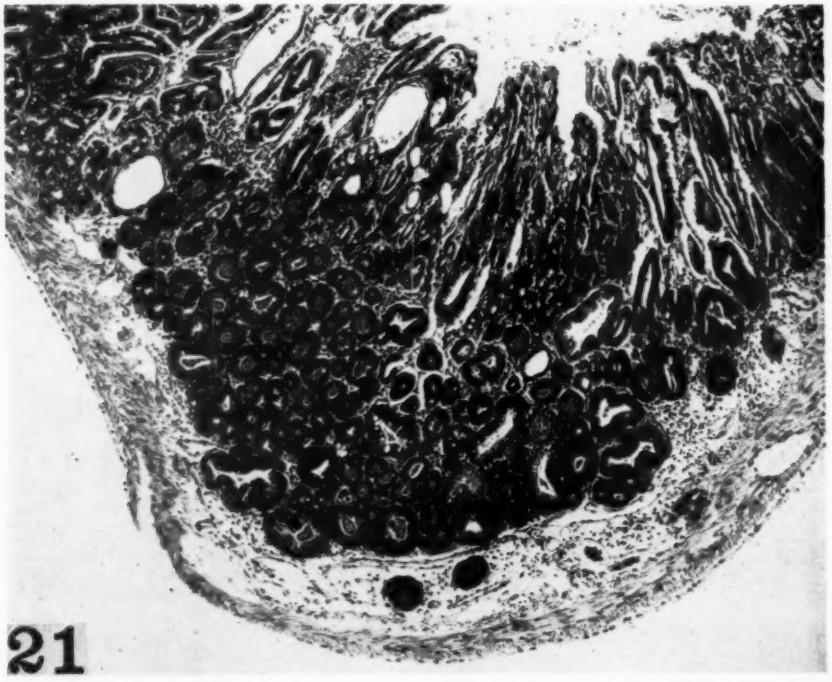


FIGURE 19.—Neoplastic change manifested almost exclusively at the basal portion of the crypts. The overlying mucosa shows degenerative changes. × 105.

FIGURE 20.—Basal glandular proliferation and degenerative changes of superficial mucosa. × 105.



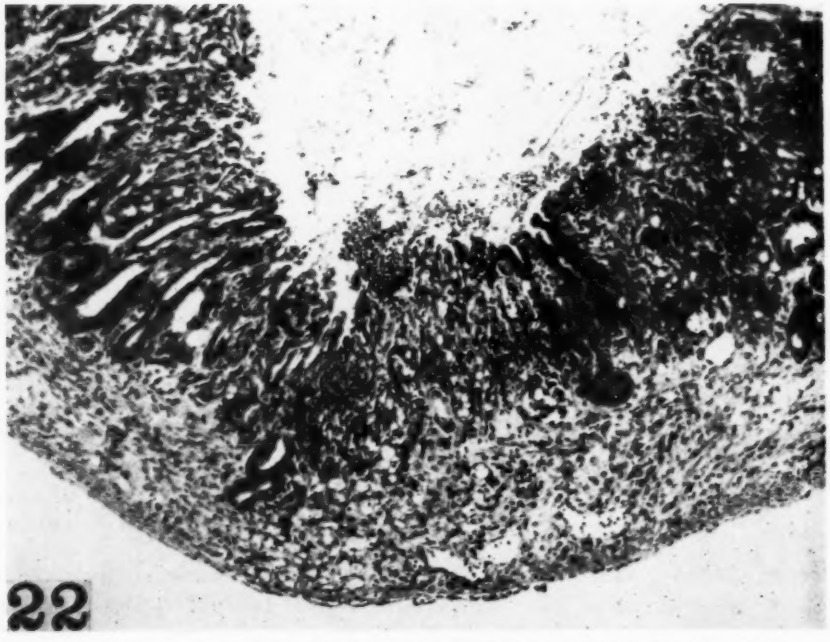
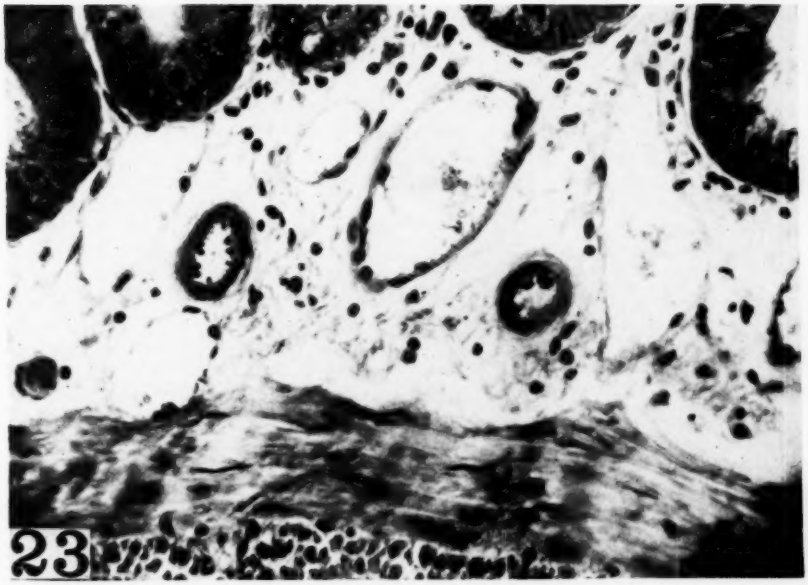


FIGURE 21.—Late basal proliferation. The superficial mucosa is atrophic. × 73.

FIGURE 22.—Muscularis infiltrated by a mucinous type of neoplastic cell. × 92.



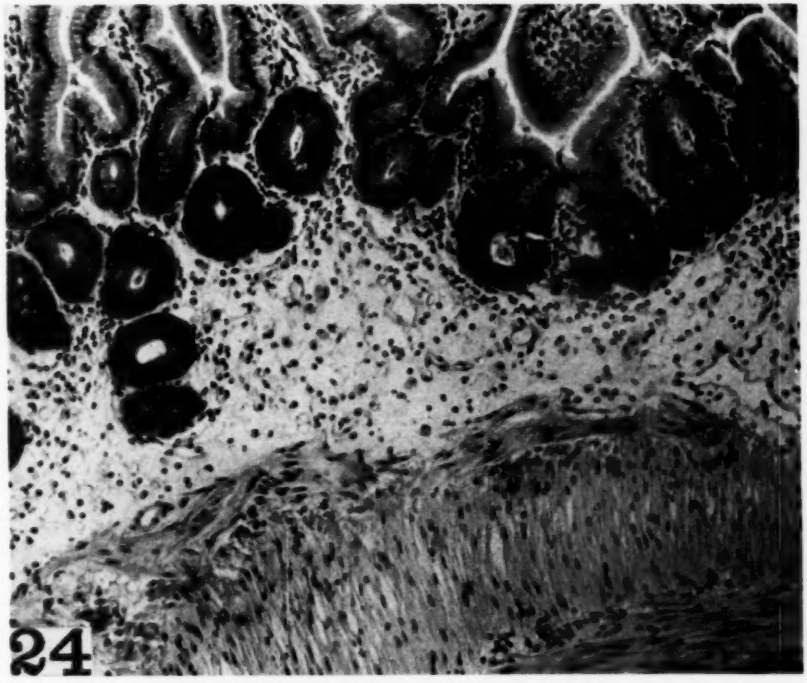


FIGURE 23.—Lamina propria, edematous, sparsely infiltrated with inflammatory cells, and containing numerous dilated lymphatic vessels. × 260.

FIGURE 24.—Lamina propria showing cellular composition and poorly stained fibrillar material. × 160.

tain engulfed lipoid, pigment, and nuclear debris. The focal inflammatory infiltrations are often concentrated about varying-sized, sharply outlined, rounded, or bottle-shaped necroses situated in the lamina propria, submucosa, muscularis mucosae, and muscularis (figs. 25, 26). Thrombi are occasionally noted in the vessels of the lamina propria and the submucosa but are not definitely correlated with the occurrence of focal areas of necrosis. Not infrequently a focus of inflammatory or lymphoid cells appears to spearhead the deep infiltration of hyperplastic glands into the submucosa and muscularis.

The reticulum and collagen fibers of the edematous lamina propria are numerically decreased, loosely arranged, more or less detached, widely and irregularly spaced, and not oriented in relation to the surface, but their long axis appears to run in different directions. The stromal cells are largely rounded or young spindle-shaped forms and show a disproportionate increase over fibrils. Some of the fibroblasts possess bipolar processes, which in turn may be branched and at times stain positively for fibroglia in the phosphotungstic acid-hematoxylin preparations. The fibrillar material itself appears degenerated and stains irregularly pale and acidophilic. Between and below the crypts, the appearance is that of a fibrinoid type of degeneration. With van Gieson stain, the fibrillar material fails to take a sharp red stain as does the rest of the collagen: with the reticulum stain it stains darkly but is split and frayed and does not have the sharply etched, coal-black pattern of normal reticulum or the brownish, wavy appearance of normal collagen. With Mallory's aniline blue stain the fibrillar material is a dull, faintly stained, blue color in contrast with the sharp-staining, deep, brilliant-blue color of normal collagen. The stroma of the tips of the villi is swollen, granular, stains basically with the hematoxylin and eosin stain, and appears degenerated. No elastic fibrils are demonstrable.

Surrounding each of the newly proliferated glands at the base of the crypts or in the midmucosa, there is a sharply etched basement membrane of reticulum. Around the original glands in the superficial mucosa, the reticulum of the basement membrane stains poorly, being frayed and absent in areas. Later these glands degenerate and disappear, and the intestinal lining is replaced by the newly proliferated glands. Although the basement membrane around each of the newly proliferated glands stains well for reticulum, the reticulum fibers that branch off from this membrane and extend out into the surrounding stroma become split and frayed and lose their sharply staining qualities (fig. 27). In a few specimens, the lamina propria of the original intestinal glands is the seat of a deposit of amyloid, which develops spontaneously in mice.

During the early developmental stage of carcinoma, the newly proliferated basal glands are separated from the muscularis by the edematous, degenerated stroma of the lamina propria and submucosa. However, once the glands have extended downward to the point where they make physical contact with the proximal muscularis, the reticulum of the musculature and that of the proliferating glands fuse, and this healthy, well-stained reticulum supports the growth during its subsequent development and infiltration through the remainder of the intestinal wall and shows a characteristic epithelial pattern (fig. 28).

The lymphatic vessels of the intestine are found dilated shortly after the mouse is placed on the hydrocarbon-emulsion regime, and this dilatation persists until, with the increase and condensation of the stroma, they become constricted and obliterated. Thus in the early stages of precancerous development there is ample physical opportunity for the proliferating glands to invade the numerous dilated lymphatic vessels in close apposition to which they lay, and then by this route to infiltrate into and through the wall of the intestine. This rarely occurs, however.[3] Instead, the lymphatics in the mucosal and submucosal stroma become obliterated and disappear and play no part in the down growth of the glands. However, once the growth has extended through the muscularis and onto the peritoneum, invasion of lymphatic vessels is readily accomplished, and tumor deposits are transported by this route to the base of the mesentery, the abdominal lymph nodes, and lungs. Whether the spread of the neoplastic glands through the muscularis is in part by way of lymphatic vessels could not be definitely determined, but most of the spread certainly appears to be by direct infiltration of the muscle tissue and not by way of lymphatic vessels.

In some specimens the neoplastic glands apparently rapidly infiltrate through the muscular coats (fig. 29). In other specimens, the inner circular layer of muscle acts as a barrier or at least offers resistance to the infiltrating neoplasm (fig. 30). The acini then flatten out, grow laterally in either direction, and later may penetrate at a point where the muscularis is interrupted by the passage of a blood vessel and its accompanying connective tissue. This is more often the case when the circular muscularis has failed to proliferate, and the polarity of its cells remains undisturbed. Following infiltration of all coats of the intestinal wall, the lateral margins of the interrupted muscularis occupy a position at right angles on either side of

[3] White and Stewart (6) illustrated a case of carcinoma of the small intestine with metastasis. The local lesion showed extensive permeation of the mucosal lymphatic vessels.

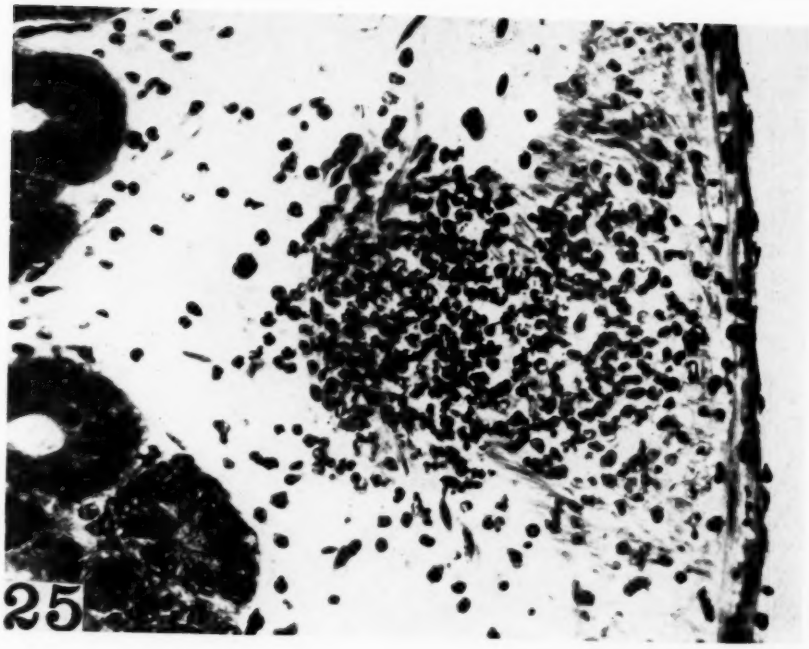
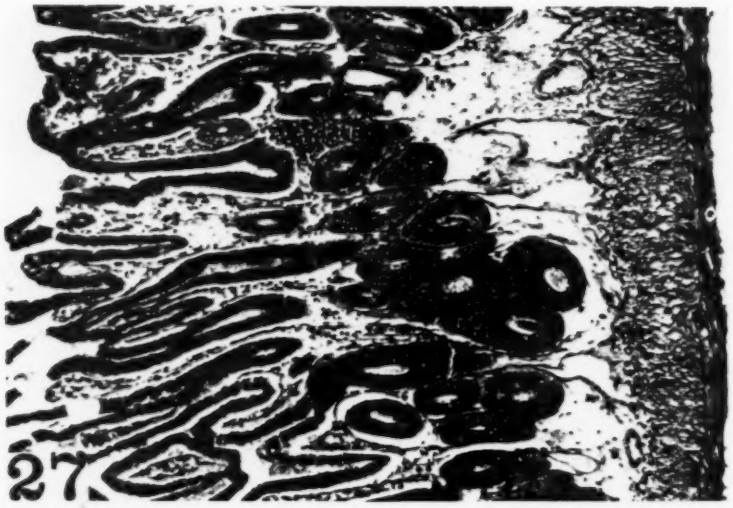


FIGURE 25.—Focal inflammatory lesion involving the muscularis and lamina propria. × 240.

FIGURE 26.—A bottle-shaped area of necrosis. × 75.



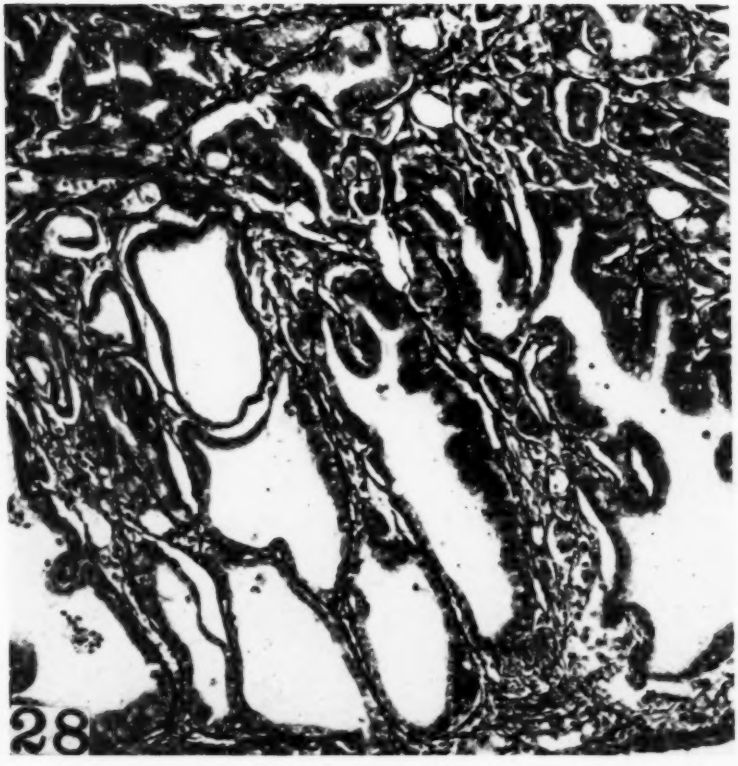
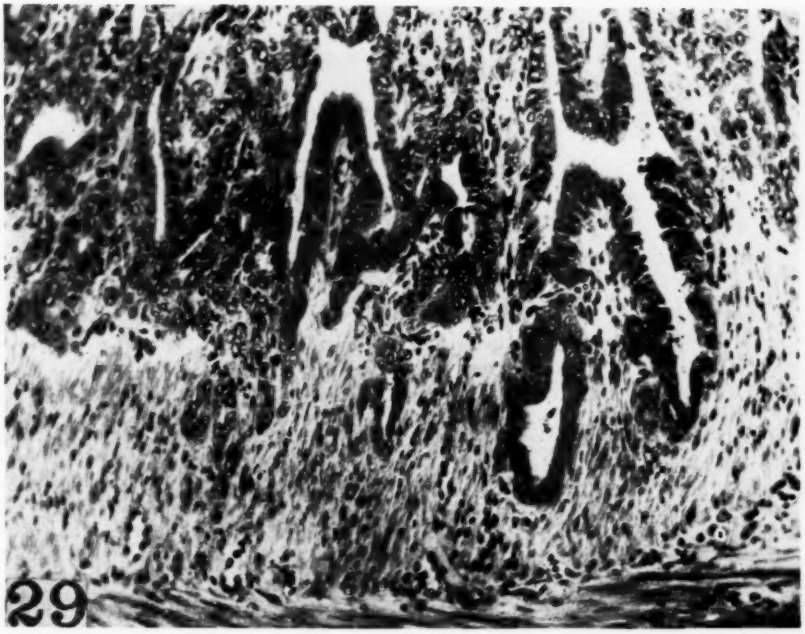


FIGURE 27.—Intestine stained for reticulum. The reticulum of the lamina propria and that about the superficial glands stains poorly in contrast with the well-stained basement membrane of the newly proliferated basal glands. × 120.

FIGURE 28.—Area of carcinoma stained for reticulum. × 160.



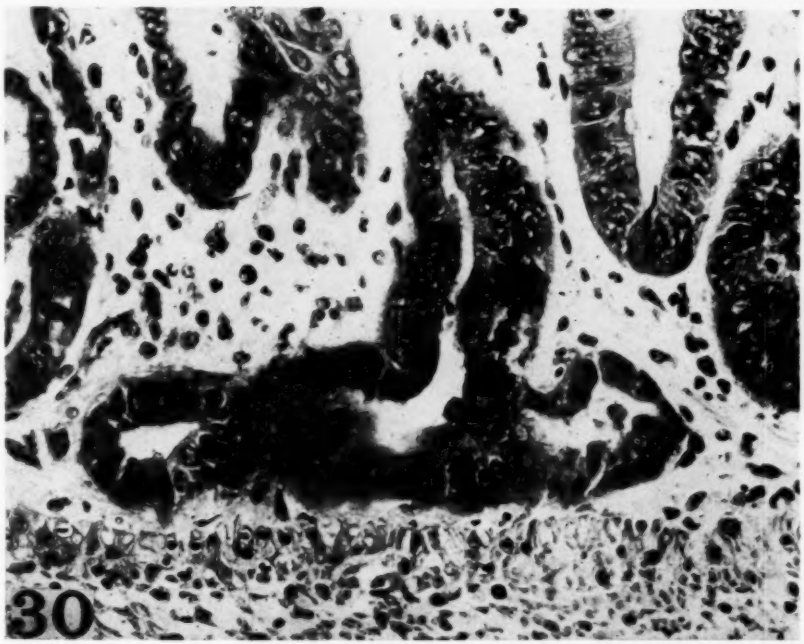


FIGURE 29.—Change in polarity of the smooth muscle cells in the inner layer of the muscularis coincident with the glandular infiltration. × 240.

FIGURE 30.—Junction between muscularis and submucosa. An atypical acinus failing to infiltrate directly has flattened out and grown parallel with the muscle coat. × 385.

the tumor occasionally reaching almost to the surface of the intestine (fig. 31), as though retracted sharply upward.

Other changes occur in the smooth muscle of the gut wall (figs. 32, 33, 34). The first of these is early tenting of the muscularis below the site of the umbilicated lesion (fig. 9). This is followed by fusion of the submucosa, muscularis mucosae, and circular layer of the muscularis coincidental with the infiltration of the neoplasm. Due to a combination of inflammatory reaction and hypertrophy and numerical increase in smooth muscle cells, the circular layer of the muscularis becomes thickened (figs. 13, 14, 15). The innermost smooth muscle cells of this coat show a change in polarity so that many of them come to be arranged perpendicular to the bowel wall. Small nodular proliferations of smooth muscle cells, positively identified by their myoglia fibrils in the phosphotungstic acid-hematoxylin preparation, curve upwards in thin filaments into the overlying stroma about the infiltrating glands. Although the role of these smooth-muscle-cell proliferations in the histogenesis of the carcinoma is not clearly evident, they are not neoplastic, for no leiomyomas or leiomyosarcomas have developed.

One route for the introduction of infection deep into the intestinal wall is by way of the invading glands (fig. 31). In the lumina of these infiltrated glands, bacterial forms and yeastlike organisms are frequently identified among the debris of leukocytes, desquamated epithelium, and secretory products of the epithelial cells. Some of the tubular glands situated deeply in the subserosa and muscularis open into the lumen of the intestine, thus accounting for the direct introduction of infection. The ulceration regularly associated with the early developing carcinomas also contributes to inflammatory changes and fibrosis.

On the peritoneum opposite the precancerous lesion, an area of chronic granulation tissue and fibrosis frequently develops (figs. 35, 36). This contains an abundance of well-stained collagen and reticulum. This serosal thickening is often seen early, even before the muscularis is invaded by cancer, and it increases in amount coincident with the extension of the glands through the muscularis. There is invariably a more or less intense inflammatory reaction, sometimes amounting to abscess formation, particularly when the overlying stroma of the neoplastic lesion is heavily infected. Deep areas of necrosis and fissures, often bottle-shaped, with associated degenerative changes and necrosis, extend down from the mucosal surface to near the serosa (fig. 26).

In a few cases, serosal plaques are composed of sheets of neoplastic-appearing cells, which resemble serosal cells and which extend into the attached mesentery for a short distance and occasionally invade mesenteric lymphatics. When a developing carcinoma overlays such an area, one cannot be sure that some of the atypical cells are not infiltrating cancer. However, serosal plaques of this type are observed unassociated with intestinal carcinoma. The possibility that these peritoneal foci may represent early malignant endotheliomas is

FIGURE 31.—Illustration of one method by which infection gains access to the deep portion of a carcinoma; fortuitous section showing continuity between a deeply placed neoplastic crypt and the lumen of the intestine. Many of the circumscribed, pale areas in the deeper portion of the growth are infected tumor acini which show desquamation of the lining cells. The muscularis is interrupted at both margins of the growth. × 32.

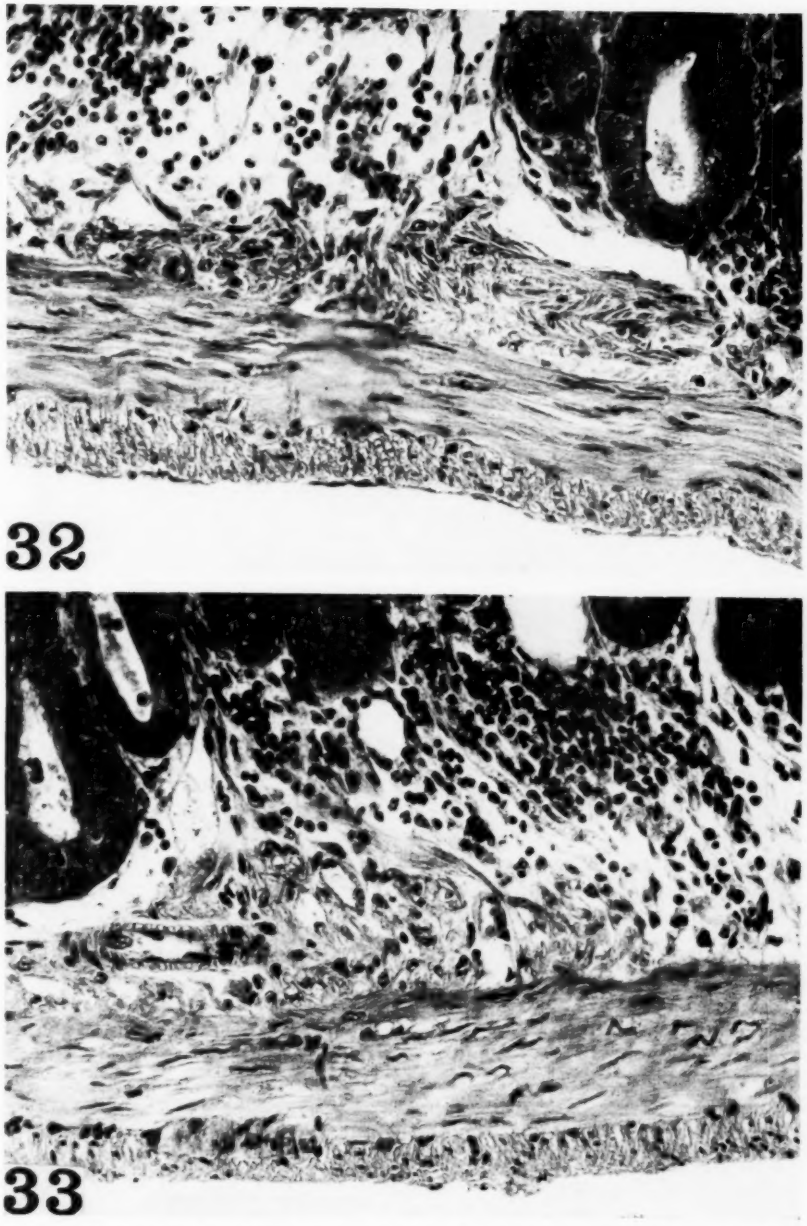


Figure 32.—Small nodular area of proliferating smooth muscle cells. × 285.

Figure 33.—Upward growths of smooth muscle cells coincident with the downward proliferation of the basal glands. × 210.

FIGURE 34.—Myoglia fibrils in proliferating muscle. Phosphotungstic acid-hematoxylin preparation, oil immersion lens. Circa × 1200.

strengthened by the fact that a number of proved malignant endotheliomas of the peritoneum have been observed under the conditions of oral administration of carcinogenic hydrocarbons (Lorenz and Stewart (*4a*) and White and Stewart (*6*)). However, the material at hand is insufficient for a study of the histogenesis of this primary malignant serosal tumor.

In several cases a carcinoma has been observed developing in close relation to an intestinal lymphoid nodule (figs. 37, 38, 39, 40). Various stages from early neoplastic infiltration to varying degrees of replacement of the lymphoid tissue by cancer have been noted.

DISCUSSION

It is virtually impossible to compare the precancerous lesions of the small intestine of mice with those of man, because with the low incidence of carcinoma of the small intestine in human beings (Stewart et al. (*7, 8, 9*) Nickerson et al. (*10*), Raiford (*11*)), there has been little or no opportunity to study the histogenesis of cancer in this location. The only precancerous lesions of the intestine of man that have been studied to any extent are those of the rectum, rectosigmoid, and lower sigmoid. These areas are available for direct inspection by proctoscopic examination, and hence from time to time lesions are observed which are suspected of being early carcinomas and are excised and subjected to histologic examination (Lockhart-Mummery (*12; 13, p. 284*), Dukes (*14–16*), Bacon (*17, p. 571*), and Daniels (*18, 19*)). Earlier studies of this type were based largely on necropsy findings (Hauser (*20–22*), Versé (*23*)). More recently, the mucosa of segments of the large intestine, removed for carcinoma, has been subjected to careful gross and microscopic examination; and studies have been reported purporting to show that precancerous lesions are frequently present in such locations (Bargen, Cromar, and Dixon (*24, 25*)). Cain and Bensaude (*26*) after examining the structure of a series of small cancers, concluded that such growths can be divided into two groups: (1) Those arising in adenomatous tissue; and (2) those occurring in apparently healthy mucous membrane. It is now generally conceded that rectal adenomatous polyps constitute the most dangerous as well as the most common predisposing factor in the development of rectal adenocarcinoma. Fitzgibbon and Rankin (*27, 28*) were able to trace 22 of 24 cancers of the large intestine directly back to polyps, and Swinton and Warren (*29*) were able to demonstrate that 14 percent of their series of malignant tumors involving the colon had arisen in mucosal polyps.

The problem was reviewed by Hellwig (*30*). The intestinal adenomas are often numerous and separated from one another by normal mucosa. The bowel may remain in this condition for many years, or at any stage of the epithelial proliferations the epithelium may abruptly assume those invasive properties recognized clinically and histologically as cancer. The incidence of malignancy in multiple polyposis is generally accepted as being from 40 to 50 percent. But according to Bargen (*24, 25*) and Rankin (*27, 28*), it is impossible from histologic and clinical examination to predict whether or not a given polyp will become malignant. While most of the authors did not have the opportunity actually to observe a malignant transformation in a particular polyp in any case, Schmieden and Westhues (*31*) described all histologic transitional stages, from harmless adenomatous hypertrophy of the mucosa to polyps with partially undifferentiated cells leading up to fully developed adenocarcinoma. Schmieden and Westhues classified the different histologic pictures into 3 groups. The third, which alone was designated as the precarcinomatous stage, is characterized by invasion of the pedicle and perfora-

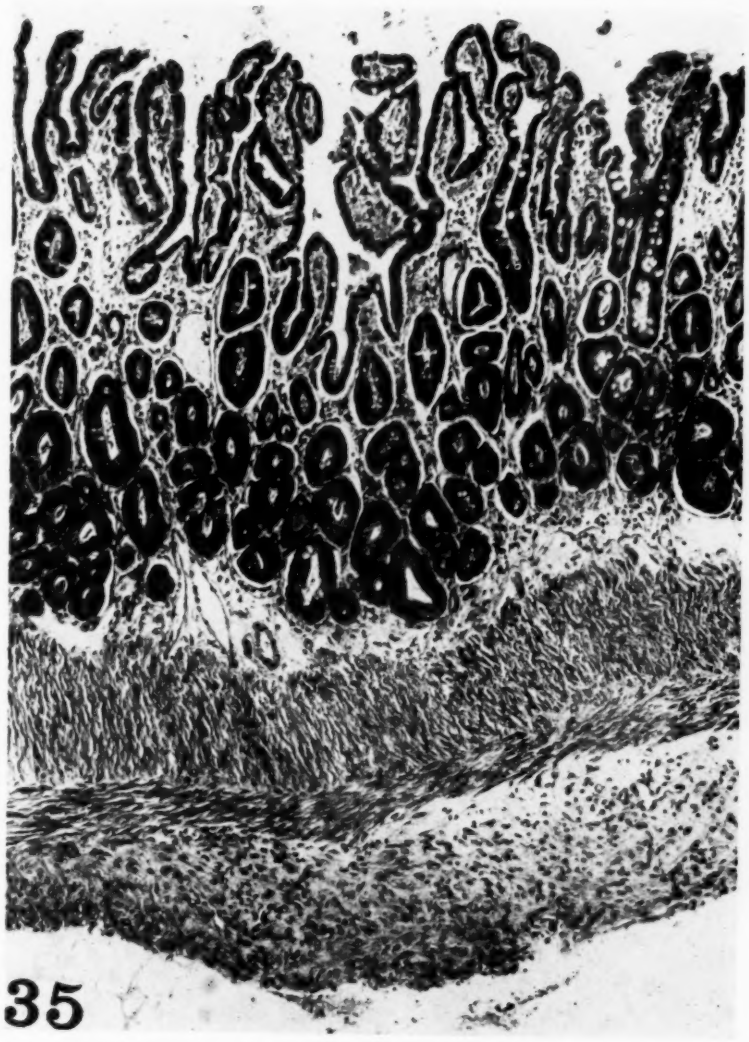


FIGURE 35.—Serosal plaque beneath precancerous lesion. × 120.

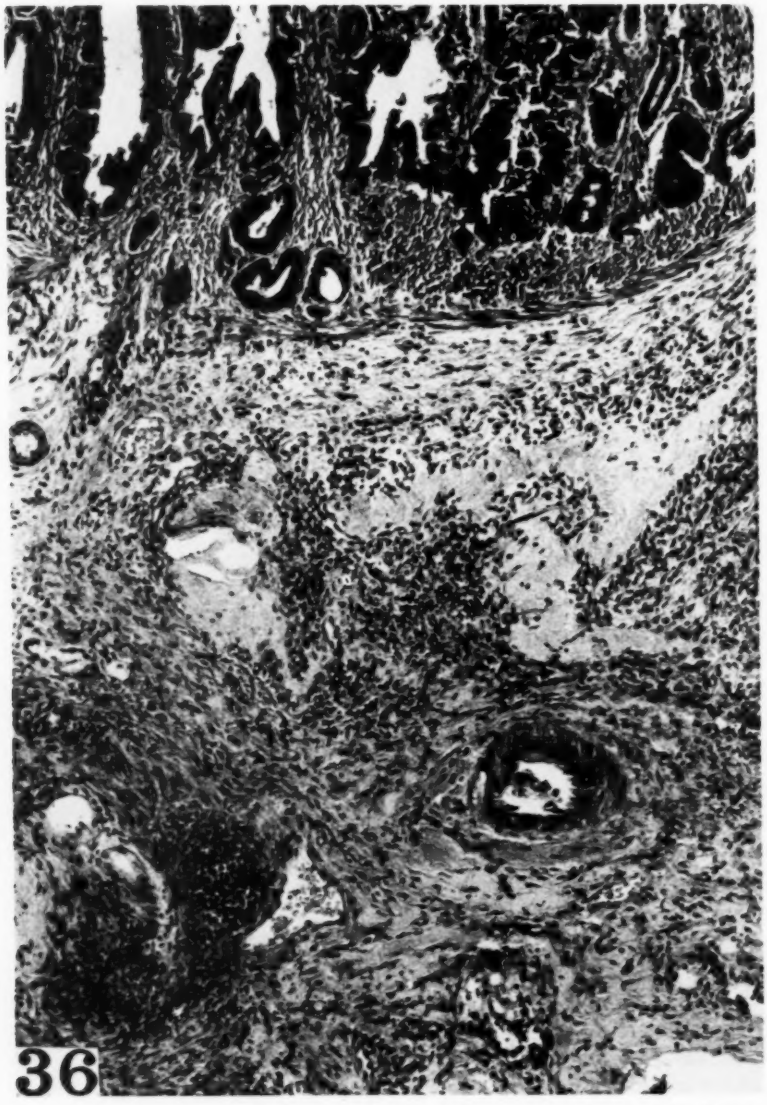
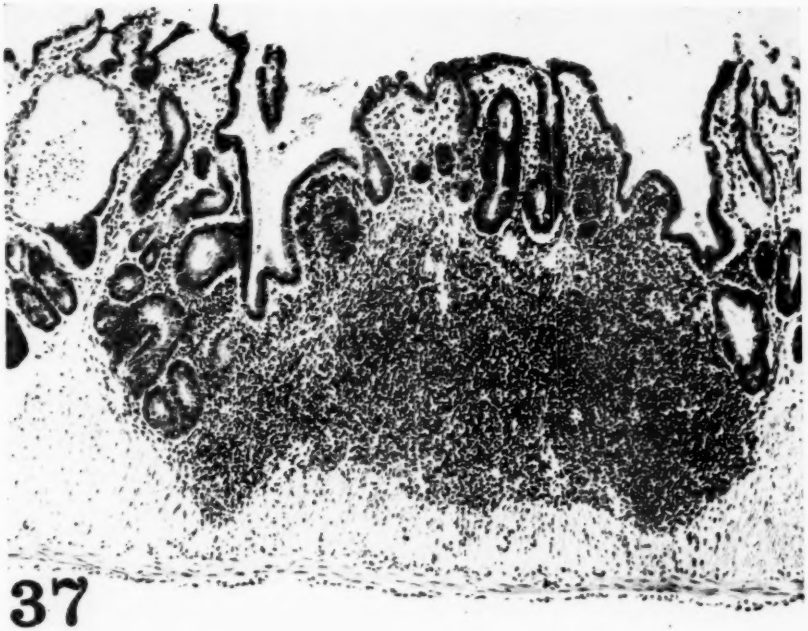


FIGURE 36.—Precancerous lesion, with serosal plaque underneath showing large areas of hyaline degeneration. × 130.



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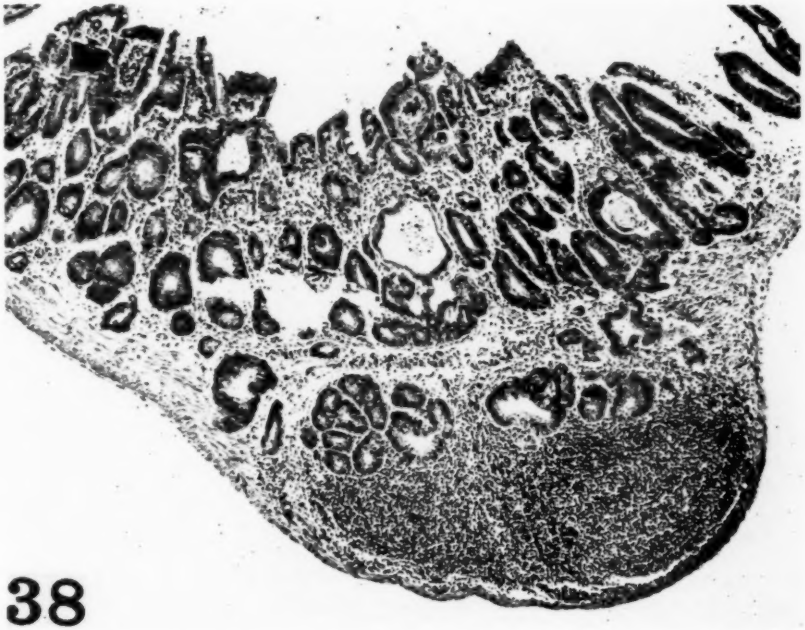

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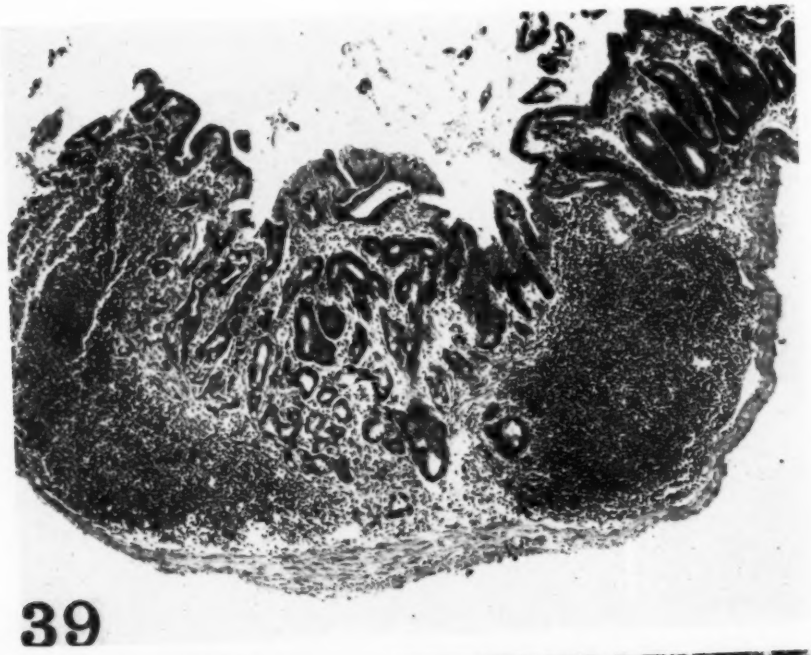
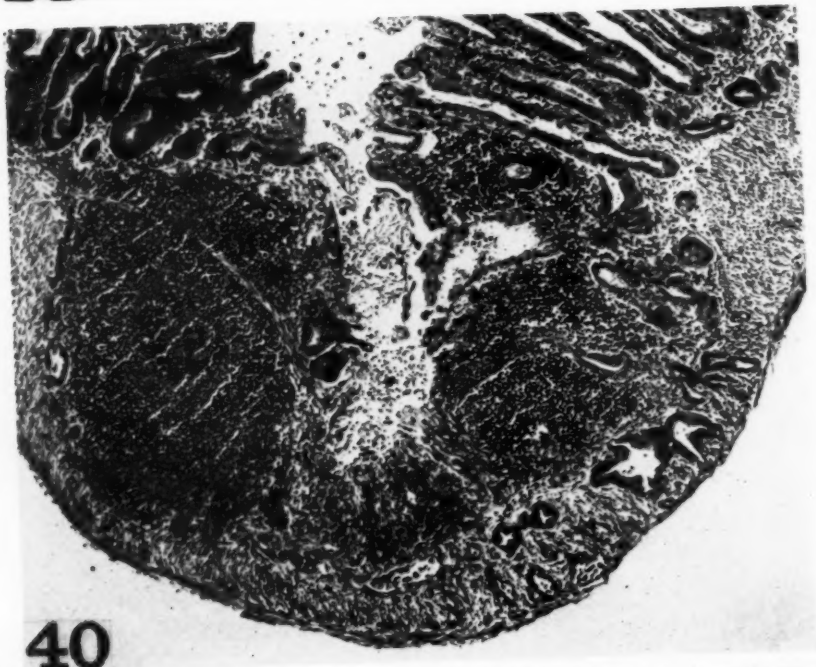
FIGURES 37 and 38.—Precancerous changes at the site of lymphoid follicle. × 105 and × 87, respectively.





FIGURES 39 and 40.—Neoplastic infiltration at the site of lymphoid follicle. × 80 and × 96, respectively.

tion of the muscular layer, but there arises the question whether this third stage with definite signs of malignancy should not be called carcinoma. Westhues' classification of intestinal polyps into 3 groups was accepted by Fitzgibbon and Rankin (*27*). In the tumors belonging to their third group, the definite precancerous lesions, the cells could not be distinguished morphologically from those seen in outright cancer.

Feyrter (*32*) examined at autopsy the large intestine of 1,110 patients over 20 years of age and found adenomatous proliferations in the colon in about one-third of them. The total number of polyps found was about 1,000, but only 6 of these were carcinomatous, and 4 were suspicious for malignancy. Of great interest is the case reported by Hullsiek (*33*) of multiple polyposis of the colon, in which the first biopsy did not show any histologic evidence of malignancy. Two years later an adenocarcinoma was revealed by biopsy at the rectosigmoid junction. Besides this carcinoma, with no suggestion of its having arisen from a polyp, the autopsy showed no carcinomatous changes in any of the polyps that extended from the rectum up to the ileocecal valve. Observations of this kind led Stahr (*34*) to the conclusion that rectal polyps like other atypical epithelial proliferations are not necessarily true precancerous stages but can be regarded only as indicators of some kind of epithelial change that is capable eventually of producing cancer under circumstances not yet understood.

Bargen, Cromar, and Dixon (*24*) examined areas of the intestine in the neighborhood of cancers of the colon in surgical specimens and described interesting changes involving lymphoid aggregations in the submucosa, with resultant damage to the muscularis mucosae. The lymphoid aggregations, situated immediately below the epithelial layer, were variable in size and number and seemed to arise as perivascular infiltrations.

When large, the lymphoid aggregation became encapsulated, developed a well-marked secondary center, and seemed to disrupt the continuity of the muscularis mucosae. This was followed by secondary changes in the overlying epithelium consisting of an irregular proliferation of the glandular structure, with the production of atypical glands and cyst formation. These changes appeared to be secondary to and the direct result of the presence of the underlying follicles. The subsequent changes seemed to be more or less mechanical in origin. In some instances, the follicles would rupture into the lumen and the epithelium would prolapse into their substance, with the escape of the atypical glandular structures composed of primitive epithelial cells, into the loose connective tissue. This change was described by Schultze (*35*) and is often referred to as the "Schultze picture." In other areas, the lymphoid follicles ruptured into the lumen of the bowel causing ulceration. In the process of healing, epithelial cells, less differentiated and more primitive than usual, were trapped in the deeper layers at the base of the ulcer. Occasionally, a small patch of epithelium became isolated from the rest of its stratum by a circle of follicles, which enlarged progressively, coincident with destruction of the muscularis mucosae, and the epithelium became pegged down to the underlying submucosa. The surrounding muscularis then contracted elevating the enclosed tissue, thus forming the first stage of an adenomatous polyp. The lymphoid tissue at the base was slowly replaced by fibrous tissue which formed the core of the polyp. Bargen, Cromar, and Dixon (*24*) found that in other cases, there was no evidence of polyps, and they believed that cancer in these cases arose from atypical cells buried in the submucosa as a result of either ulceration or rupture of the lymph follicle. The structure of the small cancer reported by Raven (*36*) suggests this mode of origin.

In the present experiment, a number of precancerous lesions were observed which developed in relation to the lymph follicle as described and illustrated. However, it was not possible to trace the complicated series of events in the lymphoid follicles of the mouse's intestine that have been noted in man.

In the stomach of man, preinvasive carcinoma occurs in the form of a polyp showing malignant cytological alterations (Borrmann (*37*)) and as a nonpolypoid form, very prone to ulceration, which occurs in the prepyloric region sometimes associated with chronic gastritis (Gutmann, Seneque, Bertrand, and Beauregard (*38*), and Ewing (*39; 40, pp. 684 743*). An apparently third type of lesion first observed by Stromeyer (*41*) and Moszkowicz (*42*), was described in detail by Mallory (*43*), who called it carcinoma "in situ." With respect to the relationship of peptic ulceration and carcinoma of the stomach, estimations of the frequency with which this is found vary from 1 percent (Borrmann (*37*)) to 71 percent (Wilson and MacCarty (*44*)). To say the least, the evidence purporting to indicate that the peptic ulcer is a precancerous lesion of the stomach is far from convincing.

The present experimental work bears no relationship to the cancers that develop from polyps in either the intestine or stomach of man. Very few primary tumors of the colon have appeared in the mice of this experiment. No polyps were observed in the small intestines of the experimental mice, hence this lesion, so common in man, played no role in the histogenesis of the malignant neoplasms under consideration. Only a few polyps were observed in the colon, and these are consid-

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ered probably spontaneous (fig. 41). Nothing of the nature of a peptic ulcer was ever observed as a precancerous intestinal lesion in the mice. With respect to the lesions of carcinoma in situ in the stomach as described by Mallory (*43*), there were points of similarity and dissimilarity. In both, the mucosa showed changes histologically resembling those of malignant neoplasm with numerous and atypical mitoses widely distributed in the area of involvement. In the human stomachs, the lesion of carcinoma in situ involved the entire thickness of the mucous membrane but did not extend beyond these limits, did not involve the base of the mucosa, and showed only limited infiltration in one case examined by Mallory. In the experimental material, the early neoplastic change in the intestine showed a predilection for the midportion of the mucosa and the basal glands, and there was infiltration from the very beginning. In Mallory's cases, the gastric carcinoma in situ showed either no ulceration or else the deep type of peptic excavation, both of which are quite unlike the umbilicated lesion in mice, which goes on to later frank ulceration. The photomicrographs of the carcinoma in situ of Mallory's cases resemble more the gastric adenomatous lesion of strain I mice (Stewart and Andervont (*45*)) than they do the induced precancerous lesions of the small intestines of mice fed the carcinogenic hydrocarbons. Strain I mice spontaneously develop a hyperplastic adenomatous lesion of the stomach, and occasionally atypical mucosal glands are found displaced in the deeper layers of the gastric wall and even in the blood vessels. However, there is no definite invasion as in cancer, and the lesion does not metastasize and is not considered malignant.

The studies of the histogenesis of early cancer of the stomach and intestines in man leaves a gap between the ordinary condition of inflammatory overgrowth and definite carcinoma. This gap appears to be bridged, if at all, by a rather sharp morphologic change which becomes established rapidly. There has always been uncertainty, or at least the investigators in this field could never be entirely convinced that the lesions which they described as early cancer would inevitably progress into cancer. The experimental field on the other hand is not beset with this disadvantage, because it has been possible to kill and autopsy animals ingesting the carcinogenic hydrocarbons at regular intervals and compare the changes observed in them with those in the controls, and the experiment is so arranged as to yield a high incidence of intestinal carcinoma. Hence the experimental material is not subject to the same criticism and objections that have always been raised in connection with studies of the precancerous lesions of the gastrointestinal tract in man.

Many observers have stated their belief in one or more of several theories, namely, that malignant tumors may originate from one circumscribed focus, possibly from one cell, and then enlarge by the expansive growth of this solitary focus or that they may develop over large surfaces simultaneously or successively so that in addition to enlarging by expansion from a central focus they may enlarge through confluence and apposition. In the present study, the origin of none of the cancers can be traced to a change in a single cell. The earliest departure from normal observed involved many cells in what were distinctly separate foci, although these foci were usually confined to

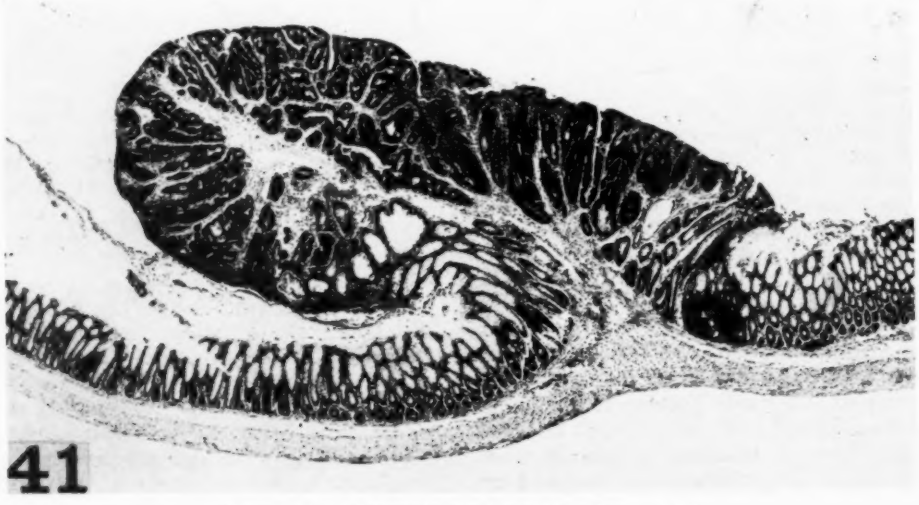

FIGURE 41.—Adenomatous polyp of the colon. × 27.

a relatively small area. As stated in the description and as illustrated, the earliest neoplastic change observed began in the midmucosa or in the crypts of several adjacent glands, which simultaneously grew downward over an area that varied in width and breadth in different specimens, depending upon whether there was a relatively small, umbilicated focus or a more diffuse, multicentric, "garden hose" type of lesion. It is inconceivable, however, that any of these originated in a single cell and that the entire tumor developed from that solitary single-cell focus. It is possible that the change may have begun first in one cell or one group of cells, and then a few hours or days later another group became involved independently and so on; but certainly the histologic distribution of the neoplastic change covers too extensive an area and involves too many otherwise intact crypts for it to be accounted for on the basis of a spread from a single-cell focus of neoplasia.

All the various types of cells composing intestinal mucosa were involved at one time or another in the neoplastic process and often in varying degree in different precancerous lesions. Most of the neoplastic lesions were composed of undifferentiated glandular cells often reproducing acini, probably derived from the principal cells of the mucosa. In many cases, however, both goblet cells and Paneth cells were numerous, and some of the fully developed local tumors and their metastases were composed almost exclusively of either of these two types. Dunn and Kessel (*46*) reported Paneth cells in two carcinomas of the small intestine, one in a mouse and the other in the rat. In the present study, signet-ring carcinomas were rather frequent . With respect to the argentaffin cells, these frequently appeared numerous in the silver preparations of the developing tumor, but the methods of fixation and staining employed were not especially designed for a study of these cells so that their exact role in the neoplastic process and their relationship to other cells of the proliferating mucosa, paricularly to the goblet cells, remains to be studied by the methods proposed by Popoff (*5*). None of the fully developed tumors or their metastases appeared to be of the type of the argentaffin tumor. However, since these tumors do occur spontaneously in the small intestine in man, one would not be surprised to find them induced under the conditions of this experiment.

There has always been a question regarding the marginal spread of malignant tumors, particularly those involving the stomach and intestines (Borrmann (*37*)). Progressive enlargement of the neoplasm has been accounted for on the basis that the margin undergoes malignant conversion or that the neoplastic cells recurrently infiltrate the borders and thus account for the increasing diameter of the growth. On the basis of the present observations in experimental mice, both of these mechanisms seem to play a role. In an early umbilicated lesion which is evolving into cancer, the histologic picture suggesting neoplastic changes covers a wider area than is suggested grossly and certainly goes beyond the borders of this small lesion. These changes continue to progress during the further development of the precancerous lesion and account for enlargement of the lateral limits of the growth to a large extent. At the same time, the lesion expands by central proliferation and the neoplastic cells infiltrate into the mucosa laterally, as well as deeply, and mushroom out onto the surface of the intestine forming a plaquelike lesion. The "garden hose" type of lesion is formed by the confluence of several adjacent neoplasms or precancerous foci, an example of multicentric tumor formation. Why this does not occur more frequently in this type of experiment is difficult to say, for certainly large segments of the intestinal mucosa are exposed to the carcinogenic effects of the hydrocarbon. In most instances, however, the size of the tumor is limited, and a continuous neoplastic change at the periphery of the growth does not take place beyond certain limits.

The two points of predilection for beginning growth of intestinal tumors are the midportion of the mucosa and the base of the crypts. The superficial portion of the mucosa undergoes degeneration and is ultimately exfoliated or lost by ulceration and replaced by the newly regenerated, deeply placed, neoplastic glands. This change takes place despite the fact that it is logical to believe that the superficial mucosa is more exposed to the action of the ingested carcinogen than are the deeper portions. Many years ago Schaper and Cohen (*47*) made a similar observation in early cancer of the stomach and intestine of man. They stated that the neoplastic process began in the midmucosa and the crypts of Lieberkühn, which act as growth centers or regeneration areas for the cellular replacement of the mucosa, analogous to the basal-cell layer of the epidermis, which may be the explanation for the observations in the present experiment.

Induced intestinal cancer in mice represents the ultimate phase of a definite series of changes, some of which are neoplastic from the beginning whereas others are nonneoplastic but apparently play some role in the evolutionary process. An example of the latter is the changes in the muscularis mucosae and the inner or circular layer of the muscularis. The latter becomes thickened immediately below the area of the developing tumor, and there is actually new proliferation of smooth-muscle cells, which form buds that extend upward in the direction of the downward-growing glands. At the same time, the polarity of the smooth-muscle

cells changes so that their long axis comes to be arranged perpendicular to the intestinal surface, whereas normally it is parallel. It is probable that this change in the circular muscularis is due, at least in part, to a change in the stress and strain of this region of a developing tumor. No doubt the contracting stroma of the developing carcinoma above exerts a pull on the bowel wall. Evidence of this is in the photomicrographs, showing tenting, or a tendency toward invagination of the muscularis mucosae, beneath an early neoplastic mucosal focus. It is natural to expect that this invaginating process once commenced will continue and be increased by peristalsis and will be further accelerated by the continued contraction of the stroma as the latter becomes more abundant to support the growing tumor. The belief that some explanation like the one just cited accounts for the phenomena observed in the smooth muscle of the intestine rather than that these changes are in response to the carcinogenic agent, is strengthened by the fact that neoplasms of smooth-muscle-cell origin were not induced in these experiments.

The histogenesis of experimental neoplasms has beeen studied most extensively in the skin and subcutaneous tissue. Destruction of collagenous fibers of the derma under the influence of carcinogenic agents has been reported ever since tar was first used to produce epithelial neoplasms (Thiersch cited by Woglom (*48*)). Its significance in the development of experimental tumors has been discussed by Wolbach (*49, 50*), and Orr (*51*). Howes (*52*) in particular studied the problem extensively. He found that the carcinogens, in contrast with other irritant but noncarcinogenic substances applied to the skin, are unique in that they destroy the derma and render it incapable of regeneration, while leaving epithelial cells capable of proliferating. Methylcholanthrene when implanted deeply in the subepithelial connective tissues likewise destroys collagen and reticulum fibers, leaving fibroblasts capable of proliferating but incapable of reforming reticulum and collagenous fibers. Thus the fibroblasts and epithelial cells are left hyperplastic because of injury and are free to proliferate because of the lack of properly organized intercellular substance.

During the progressive development of the precancerous intestinal lesions of mice, degenerative changes occur in the fibrillar material, collagen, and reticulum, of the lamina propria and submucosa including the basement membrane of the original mucosal glands. However, as soon as the neoplastic glands begin to proliferate they immediately develop a well-stained, healthy reticulum for a basement membrane which accompanies the neoplastic proliferation of glands, through all stages of tumor development, and which is carried over into the fully formed carcinoma, where it has an epithelial pattern. Mo of the mice of this experiment were kept on th hydrocarbon-emulsion regimen with only bri interruptions right up to the time when they we killed for autopsy. Whether the degenerati changes observed in the original collagen an reticulum are related to the continued administr tion of the emulsion could be determined by settin up new experiments in which this factor is co trolled. After sufficient hydrocarbon has bee administered to insure tumor induction, the emu sion could be replaced by plain drinking water.

SUMMARY

The early morphologic changes which eventua in carcinoma of the small intestine in mice trea orally with the carcinogenic hydrocarbons methylcholanthrene and 1,2,5,6-dibenzanthrace are described and discussed.

Grossly, the earliest precancerous lesion detecte consisted of a circumscribed area 1 to 4 mm. in d ameter, with a central umbilicate depression, su rounded by a slightly elevated, smooth, firm, ci cular border, which may be single or mul widely distributed from the pylorus to the cecal junction but is most apt to occur at a p 15 cm. from the pylorus. Segments of small i testine diffusely thickened over an area 3 to 9 c showed multicentric precancerous lesions. N polypoid lesions were observed.

Microscopically, the earliest precancero changes involved several groups of epithelial cel and occurred in the midmucosa and at the base the glands. The usual cytologic characteristics malignant neoplasm were observed from the b ginning of the earliest changes noted histolog cally. The neoplastic epithelium progressively in filtrated all coats of the intestinal wall.

The majority of the precancerous lesions an early carcinomas were composed of undifferent ated glandular cells derived from the mucosa. I many of the specimens there were numerous sig net-ring cells and Paneth cells also.

Degenerative changes occurred in the collage and reticulum of the lamina propria and submu cosa: but the newly proliferated neoplastic cel and glands developing in the midmucosa and mor deeply from the crypts, possessed a healthy, well stained reticulum from their inception, which per sisted in the fully formed carcinoma.

Changes occurred in the smooth muscle cells o the muscularis mucosae and inner circular layer o the muscularis, which were not neoplastic, bu which were considered secondary to stress an traction of the developing cancer.

Several precancerous lesions were observed de veloping in relation to the intestinal lymp follicles.

A comparison is made between the intestina precancerous lesions of man and mouse.

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ENZYMATIC DESAMIDATION OF GLUTAMINE IN THE PRESENCE OF PYRUVATE FURNISHED BY CONCOMITANT REACTIONS[1]

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INTRODUCTION

The desamidation of glutamine and of asparagine by aqueous extracts of several kinds of animal tissues is considerably accelerated when sodium pyruvate is added to the mixture (*1*). Under the conditions employed, the acceleration in desamidation becomes appreciable when the molarity of the pyruvate relative to that of the glutamine is about 0.1 to 1, reaches a maximum when this ratio is about 2 to 1, and falls off again as this ratio is further increased. The pyruvate apparently plays a role of cosubstrate in the reaction, for it is not consumed, and its final concentration in digests which originally contained glutamine is the same as in digests which lack this amino acid amide. The increase in ammonia N in digests of rat-liver extract with glutamine and pyruvate is accompanied by a corresponding decrease in amide N.

It was considered of interest to study this acceleration of desamidation by pyruvate furnished simultaneously by concomitant reactions in the same digest. Peptides of cystine (*2*), and of α—aminoacrylic acid (dehydroalanine) (*2, 3*), when incubated with fresh aqueous extracts of rat liver, are rapidly hydrolyzed to products which include ammonia and pyruvic acid. If glutamine is also present in such digests, the amount of ammonia N produced should be considerably increased, above that due to the sum of the glutamine alone and of the peptide digest alone. Under certain experimental conditions we have found this to be indeed the case.

It is evident, however, that such an increase in ammonia N production in digests of the peptides plus glutamine may, in the absence of complete hydrolysis of either the peptides or of glutamine, be susceptible to at least three interpretations. These are (1) that the extra ammonia N is derived from glutamine as a result of the presence of pyruvate furnished by the hydrolysis of the peptides, in much the same way as if pure pyruvate had been added to a glutamine digest, (2) that the extra ammonia N is derived from the peptides through some catalytic effect of glutamine on the hydrolysis of the peptides, and (3) that although the extra ammonia N may in fact be derived by increased hydrolysis of glutamine, this effect is induced by the presence of the intact cystine peptide or of its degradation products other than pyruvate, such as hydrogen sulfide. The data presented in this paper indicate that interpretation (1) is substantially correct.

EXPERIMENTAL PROCEDURE

The livers of rats were freshly removed from animals killed by decapitation, and were ground to a paste with sand in a glass mortar. Distilled water was added to the mass, and the mixture after homogenization was lightly centrifuged to remove sand and debris. The supernatant served as the enzyme source, and unless where otherwise noted, was invariably used within a half-hour after preparation.

The glutamine used was a product of the Lederle Laboratories of the American Cyanamide Co. and was generously donated to the authors. The cystine peptides, all of which are optically active, have been described (*2, 3*). The synthesis of glycyldehydroalanine has been described by Bergmann and Grafe (*4*).[3]

The desamidation was followed by measuring at various time intervals the amount of ammonia evolved. The technique of such measurements has been described (*2*). Unless where otherwise noted the pH of the digests was invariably 6.8–6.9.

EFFECT OF CYSTINE PEPTIDES

The desamidation of glutamine alone in fresh aqueous extracts of rat liver is very slight. The splitting of peptides of cystine in such extracts

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[3] We have experienced some difficulty in the preparation of chloracetyldehydroalanine from which the glycyl peptide is derived. According to Bergmann and Grafe (*4*), chloracetyldehydroalanine is synthesized by heating a mixture of chloracetamide with pure pyruvic acid under reduced pressure. After a successful initial attempt, employing a carefully fractionated Eastman specimen of pyruvic acid, subsequent attempts to prepare chloracetyldehydroalanine were consistently unsuccessful. Unaltered chloracetamide was recovered in every case. It was considered desirable to prepare and purify our own pyruvic acid, and this material was obtained by dehydration of tartaric acid as described in Organic Syntheses (*5*). When this preparation was treated with a sample of chloracetamide which had also been prepared by methods described in Organic Syntheses a partial synthesis of chloracetyldehydroalanine was achieved. Spectrophotometric analysis of the product in the ultraviolet region of the spectrum (*6*) showed that about half consisted of the desired peptide. Since the solubility behavior of chloracetamide and of chloracetyldehydroalanine is very similar, no attempt was made to separate these components from each other, and the entire mass was treated with concentrated ammonia as described by Bergmann and Grafe. When the resulting mixture of glycine amide and glycyldehydroalanine was treated with alcohol, an easy separation was effected; for the former went into solution, and the latter precipitated. After two precipitations from aqueous solution with alcohol, glycyldehydroalanine was obtained in a pure state. The nitrogen value was 19.0 percent as compared with the theoretical value of 19.2 percent. The melting point was 191° C., as compared with the value of 192°–193° given by Bergmann and Grafe. The absorption spectrum of the new preparation in the ultraviolet was identical with that reported earlier (*6*).

results in the formation of products which include pyruvic acid and ammonia. When both glutamine and a cystine peptide are present in such extracts, the ammonia produced is greater than the sum of the ammonia produced by each. These phenomena are described in table 1.

TABLE 1.—*Desamidation of glutamine in fresh rat liver extract and in the presence of cystine peptides* [1]

Peptide	Ammonia N observed in — [2]								Increase in ammonia N in digests of glutamine+peptide over sum of both	
	Extract alone		Extract+glutamine		Extract+peptide		Extract+glutamine+peptide			
	4 hours	8 hours	4 hours	8 hours	4 hours	8 hours	4 hours	8 hours	4 hours	8 hours
Cystinyldiglycine	32	55	47	75	131	175	183	269	36	74
Cystinyldidiglycine	35	44	47	57	187	204	234	274	36	57
Diglycylcystine	37	40	42	57	192	216	263	346	66	113
Dichloracetylcystine	37	40	49	66	140	210	160	280	8	44

[1] Digests consisted of 1 cc. fresh aqueous rat liver extract equivalent to 333 mg. tissue plus 1 cc. each of aqueous substrate solutions, plus sufficient water where necessary to bring total volume to 3 cc. Concentrations of stock solution of glutamine were 2.1 mg. per cubic centimeter, of cystinyldiglycine, diglycylcystine, and dichloracetylcystine 10 mg. per cubic centimeter, and of cystinyldidiglycine 12 mg. per cubic centimeter. Digestion temperature, 37° C. Digestion periods indicated.

[2] In terms of micrograms.

The data in table 1 are given in full to indicate the calculations necessary. The ammonia produced from the peptides is of nearly the same order of magnitude for each of the peptides. In the presence of glutamine, more ammonia appears to be produced in digests containing diglycylcystine after 8 hours of digestion than with the other cystine peptides. No explanation is available at present to explain this, but in view of the relatively large quantities of ammonia measured, and the consequent errors introduced by subtraction, as well as the fact that different extracts were used in each case, we would be disinclined to emphasize any differences noted among the cystine peptides.

The ammonia N derived from the cystine peptides, corrected for the extra controls, amounts to about 10–13 micromoles in 4 hours, and to about 13–15 micromoles in 8 hours of incubation. Since the ammonia and pyruvic acid are produced from these substrates in equimolar quantities (*2*), it is possible to compare the effect of the pyruvate produced from the cystine peptides on the desamidation of glutamine with the effect elicited by known amounts of added pyruvate. From the data in table 1, the effect of 10–13 micromoles of pyruvate derived from the break-down of the cystine peptides amounts in 4 hours of incubation with glutamine and rat liver extract to an increase in ammonia N of about 2 micromoles. Under similar experimental conditions as described earlier (*1*), 10–13 micromoles of pyruvate added to a digest containing glutamine, caused an increase in 4 hours of digestion of about 5 micromoles of ammonia N. However, in the last mentioned case, the pyruvate was added to the mixture at the start of the 4-hour incubation period, whereas when the cystine peptides were used the pyruvate must have been progressively accumulating (*2*). It might be more proper, perhaps, to draw comparisons with the results on the 8-hour incubation period of the cystine peptides; and in this event, the increase of ammonia N on digestion of the cystine peptides with glutamine amounts to about 4 micromoles. It would appear, therefore, that although such comparisons may present certain hazards, it is not improbable that the effect of the pyruvate derived from the break-down of cystine peptides on the desamidation of glutamine in rat-liver extracts is of very nearly the same order of magnitude as the effect produced, under nearly similar circumstances, by the same amount of pyruvate added to a digest of glutamine in rat liver extracts.

The effect of the cystine peptides on enhancing ammonia N production in digests of glutamine is due to the pyruvate produced, and not to the other products of the break-down of these substrates, namely, glycine or hydrogen sulfide. Neither of these substances, added in the appropriate concentrations to rat-liver digests containing glutamine, altered the desamidation of the glutamine. That the effect of the cystine peptides is not due to the intact peptide as such is shown in studies on dialyzed extracts of rat liver. Such dialyzed extracts have lost the capacity to attack and degrade the cystine peptides (*2*) but nevertheless have retained the capacity to cause the production of more ammonia N from glutamine in the presence of added pyruvate (*1*). The data are given in table 2.

The data in table 2 show that in dialyzed extracts of rat liver in which the degradation of diglycylcystine is very considerably reduced, no increase in ammonia N is noted in the presence of glutamine. When pyruvate is added to such a digest of the dialyzed extract with glutamine, a considerable increase in ammonia N production results.

TABLE 2.—*Desamidation of glutamine in dialyzed and undialyzed rat-liver extracts and in the presence of diglycylcystine* [1]

Ammonia N observed in—[2]								Increase in ammonia N in digests of glutamine+peptide over sum of both	
Extract alone		Extract+glutamine		Extract+peptide		Extract+glutamine+peptide			
Dialyzed	Undialyzed	Dialyzed	Undialyzed	Dialyzed	Undialyzed	Dialyzed [3]	Undialyzed	Dialyzed [3]	Undialyzed
0	41	22	47	27	175	50	262	0	81

[1] Fresh aqueous rat-liver extract equivalent to 333 mg. tissue per cubic centimeter was divided into halves, and one-half was dialyzed for 24 hours against distilled water at 5° C. Digests consisted of 1 cc. extract plus 1 cc. each of substrate solutions, plus sufficient water where necessary to bring total volume to 3 cc. Mixtures digested for 6 hours at 37° C. Substrate concentrations as in table 1.

[2] In terms of micrograms.

[3] In such digests of dialyzed extracts, when 3.2 mg. pyruvate is substituted for the peptide, the ammonia nitrogen amounts to 117 micrograms or an increase in 95 micrograms over that value of 22 micrograms in the presence of glutamine alone. This confirms earlier findings (*1*).

AMIDE NITROGEN IN DIGESTS OF GLUTAMINE AND DIGLYCYLCYSTINE

Earlier studies had shown that the increase in ammonia N noted in digests of glutamine and pyruvate was accompanied by a corresponding decrease in amide nitrogen in such digests (*1*). The amide nitrogen was determined after a brief boiling of the digest with 1N–2N hydrochloric acid.

The method was applied with some modifications to digests of glutamine with diglycylcystine in extracts of rat liver. Under the conditions employed, ammonia is not formed from the heating of diglycylcystine with hydrochloric acid. The technique used was to set up the following four digests: (1) 3 cc. of rat-liver extract at concentration equivalent to 333 mg. tissue per cubic centimeter plus 6 cc. of water; (2) 3 cc. of the liver extract plus 3 cc. of aqueous glutamine solution at concentration of 2.1 mg. per cubic centimeter, plus 3 cc. of water; (3) 3 cc. of the liver extract plus 3 cc. of aqueous diglycylcystine solution at concentration of 10 mg. per cubic centimeter plus 3 cc. of water; and (4) 3 cc. of the liver extract plus 3 cc. of aqueous glutamine solution, plus 3 cc. of aqueous diglycylcystine solution. The digests were incubated for 8 hours at 37° C. Three cubic centimeters was then removed from each digest and the free ammonia determined in the usual manner (*1*). Another 3 cc. was removed from each digest, treated with 1 cc. of concentrated hydrochloric acid and gently boiled over a free flame for exactly 1 minute. At the end of the period of boiling, each tube was immediately inserted into an ice bath. Enough chilled 40 percent sodium hydroxide solution was added to each tube to neutralize the acid, and the mixture was analyzed for ammonia. The values found after the acid treatment, corrected for the extract blank, yield the sum of the enzymatic splitting of ammonia from the substrates and the unsplit amide nitrogen. The unsplit or residual amide nitrogen can then be calculated by difference. Such studies reveal that the increase in ammonia N found in digests of glutamine with diglycylcystine can be closely accounted for by a decrease in amide nitrogen. An example of these studies is given in table 3.

TABLE 3.—*Amide nitrogen of glutamine incubated with diglycylcystine in presence of rat liver extracts* [1]

Substrate	Ammonia N	Residual amide N
	Micrograms	*Micrograms*
Glutamine	4	[2] 204
Diglycylcystine	122	
Glutamine+diglycylcystine	[2] 311	19

[1] Details of experiment in text.

[2] Amide nitrogen of glutamine determined was 190 micrograms.

The increase in ammonia N in digests of glutamine plus diglycylcystine over the sum of the ammonia in digests of glutamine alone and of diglycylcystine alone amounts to 185 micrograms (table 3). This increase, were it to come entirely from the desamidation of glutamine, would represent nearly complete splitting of the glutamine. That this is so is shown by the data in the last column of table 3. Within the errors of measurement, and of calculations involving the subtraction of relatively large quantities, it can be noted that the amide N of glutamine alone in the liver digests can be recovered by the acid hydrolysis employed, and that the disappearance of amide nitrogen in digests of glutamine with diglycylcystine, amounting to $204-19=185$, is quantitatively accounted for by the increase in ammonia N (table 3), namely, $311-122-4=185$. This concordance in the experimental findings is undoubtedly better than should be expected from the methods employed.

Attempts to repeat these studies when glycyldehydroalanine was used in place of diglycylcystine were found to be impracticable because the former peptide when heated with hydrochloric acid yields ammonia. If we reason to some extent by analogy, however, we may assume that any increase in ammonia in digests of glutamine with glycyldehydroalanine is accomplished at the expense of the desamidation of the glutamine.

EFFECT OF GLYCYLDEHYDROALANINE

The effect of increasing concentrations of glycyldehydroalanine on the desamidation of glutamine within the same time interval is given in figure 1. The digests consisted of 1 cc. fresh aqueous rat-liver extract equivalent to 333 mg. tissue plus 1 cc. aqueous glycyldehydroalanine contain-

ing the amounts designated in figure 1 plus either 1 cc. of water or 1 cc. aqueous glutamine solution containing 2.1 mg. The incubation period was 4 hours at 37° C. With increasing concentrations of glycyldehydroalanine, the difference in ammonia produced between the digests of the peptide plus glutamine and of the peptide alone increases and approaches the value of 200 micrograms, corresponding to complete desamidation of the glutamine.

The enzymatic hydrolysis with time of similar mixtures is given in figure 2. The digests were made up as just described. At 4 hours of incubation, the increase in ammonia N with glutamine is greater at the higher glycyldehydroalanine concentration.

The enzyme which catalyzes the hydrolysis of glycyldehydroalanine to glycine, pyruvic acid, and ammonia has been termed "dehydropeptidase I" (*3*). That it is an extremely active enzyme may be noted by reference to figure 2. Complete hydrolysis of 1.8 mg. of the substrate yields 175 micrograms ammonia N, and complete hydrolysis of 3.6 mg. yields double this amount of nitrogen. From the data in figure 2, a maximum of 136 micrograms is observed in the former case, corresponding to 78 percent hydrolysis of the peptide, and a maximum of about 275 micrograms in the latter case, corresponding to a similar percent hydrolysis of the peptide. No explanation why complete hydrolysis of the peptide is not achieved under these conditions is available at the present time. The amount of pyruvate which should be present at the lower peptide concentration is about 600 micrograms, and at the higher, about 1,200 micrograms. The increase in ammonia N noted (fig. 2) in glutamine digests containing 1.8 mg. of the peptide is about 50 micrograms, and in glutamine digests containing 3.6 mg. of the peptide is about 110 micrograms. The figures may be compared with those earlier reported on the increase in desamidation of glutamine in rat-liver digests to which 620 and 1,250 micrograms of pyruvate had been added (*1*). In the former case, the increase was 72 micrograms of ammonia N, and in the latter 90 micrograms of ammonia N. The agreement in the values of the ammonia increase by glutamine desamidation for known amounts of added pyruvate and for nearly equivalent amounts of pyruvate known to appear in digests of glycyldehydroalanine is sufficiently close to warrant the assumption that such increase in digests of glutamine plus glycyldehydroalanine is due to the pyruvate which appears as a degradation product of the dehydropeptide.[4]

It may be recalled that in digests of glutamine plus glycyldehyroalanine there are at least three enzymatic mechanisms operative (1) glutaminase, (2) dehydropeptidase I, and (3) for want of a

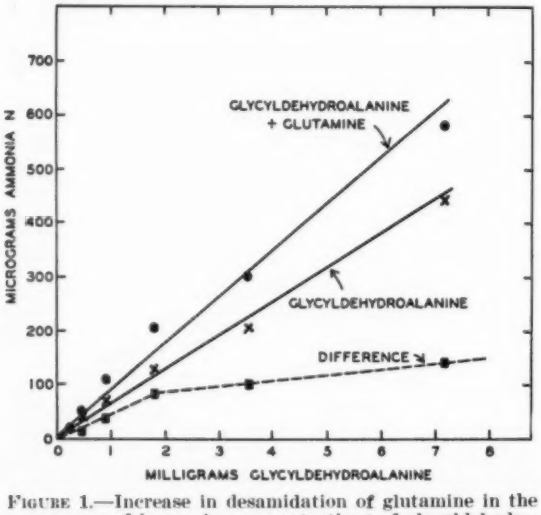

FIGURE 1.—Increase in desamidation of glutamine in the presence of increasing concentrations of glycyldehydroalanine.

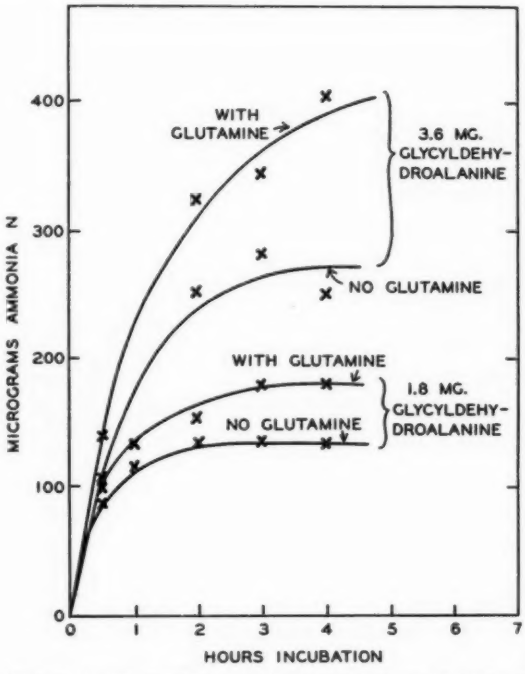

FIGURE 2.—Time course of increase in desamidation of glutamine in presence of different amounts of glycyldehydroalanine.

[4] Actual measurement of pyruvate in liver digests (*2*) containing 1.8 mg. of glycyldehydroalanine plus glutamine reveals the presence of 580 micrograms at the end of 4 hours of incubation.

better designation, what might be referred to as the "pyruvate effect." Studies were made to observe the pH–activity relationships of these three systems. Digests were based on rat liver concentrations of 333 mg. tissue per cubic centimeter. Dehydropeptidase I activity was determined with 3.6 mg. peptide per cubic centimeter aqueous stock solution, glutaminase with 2.1 mg. amino acid amide per cubic centimeter aqueous stock solution. The pyruvate effect was determined with a mixture of 1 cc. extract plus 1 cc. glutamine solution, plus 1 cc. sodium pyruvate solution containing 2.4 mg. pyruvate. Incubation period of dehydropeptidase was 2 hours, that of glutaminase and of the pyruvate effect was 4 hours, at 37° C. The buffer had no apparent effect in itself on the activity of the enzyme systems studied. The results are described in figure 3. Figure 3 shows that the pH optimum of glutaminase activity is about 8.2, similar to that already noted (*7*). The pH optimum of dehydropeptidase I and of the "pyruvate effect" is about 7.2. In acid regions of pH where glutaminase activity is minimal, both dehydropeptidase I and the pyruvate effect are still highly active. This phenomenon alone is sufficient evidence to remove the possibility that the increase in desamidation of glutamine in digests of liver with glycyldehydroalanine at pH 6.8 might be caused by an activation of glutaminase. The broad range of activity over several pH units of both dehydropeptidase and of the pyruvate effect is particularly interesting in view of the interrelation of the two postulated earlier (*1*).

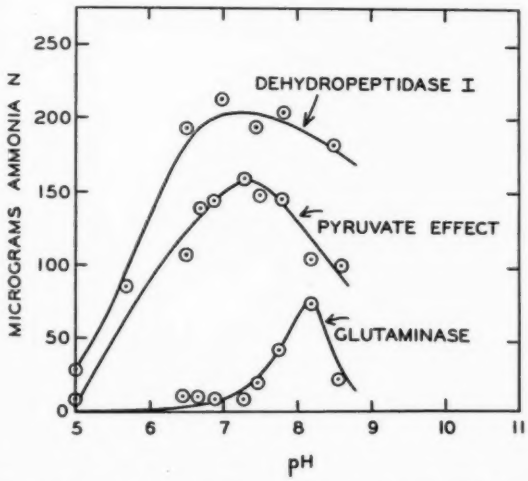

FIGURE 3.—pH-Activity curves of glutaminase, of dehydropeptidase I, and of "pyruvate effect," i. e., the difference between the values of glutamine plus pyruvate and glutamine alone. The pH of digests was set by veronal-acetate buffers and checked by the glass electrode.

DISCUSSION

It is clear that the hydrolysis of cystine peptides and of glycyldehydroalanine which furnishes pyruvic acid as one of the hydrolytic products can result, in the presence of glutamine, in an increase in desamidation of the glutamine to an extent similar to that induced by an equivalent amount of added sodium pyruvate. Just as the increase in desamidation is increased up to an optimum level by increasing amounts of pyruvate, so, too, this desamidation is increased in the presence of increasing amounts of dehydropeptide.

In an earlier report (*1*), the mechanism of the effect of pyruvate on amino acid desamidation was proposed as follows:

$$RCONH_2 + R'COCOOH \rightarrow RCON{=}C(COOH)R' \quad (1)$$

$$RCON{=}C(COOH)R' \rightarrow RCOOH + NH_3 + R'COCOOH \quad (2)$$

Thus, the first step in the reaction consists in a condensation of the amide with the keto acid to form a dehydropeptide, followed by the second step which is the splitting of the dehydropeptide by dehydropeptidase to yield the amino acid, ammonia, and the keto acid. The fact that the keto acid is not altered in concentration as a result of the reaction has been shown (*1*). According to the reactions given, dehydropeptidase is one of the reacting enzymatic systems.

The pH-activity curve of the pyruvate effect (fig. 3), which effect has been described in terms of reactions 1 and 2, has the same optimum as has dehydropeptidase, and is quite different from that of glutaminase. The maximum pyruvate effect apparently occurs at a pH of 7.2 where the activity of glutaminase is almost nil. This effectively rules out any effect of pyruvate being due to an augmentation of glutaminase activity. The reaction mechanisms 1 and 2 given may require modification or alteration as more experimental data are obtained; but at the present time, they form the most plausible working hypothesis available.

SUMMARY

Digests of diglycylcystine, cystinyldiglycine, cystinyldidiglycine, and dichloracetylcystine with rat-liver extract yield products which include ammonia and pyruvic acid. Digests of glutamine with rat-liver extract yield very little ammonia. Mixtures of the cystine peptides with glutamine in digests of rat-liver extract yield amounts of ammonia in excess of the sum of the peptide and of glutamine taken separately. The increase in ammonia in such mixtures is accounted for by the desamidation of the glutamine. The effect of the

pyruvate produced by hydrolysis of the peptides upon the desamidation of the glutamine is of the same order as that induced by the same amount of added pyruvate in a digest with glutamine.

Digests of mixtures of glycyldehydroalanine with glutamine and rat-liver extract yield more ammonia than the sum of each component taken separately. The increase is proportional, under otherwise constant conditions, to the concentration of dehydropeptide. The increase in desamidation of the glutamine occurs only in concentrated extracts of liver. The optimum activity of glutaminase occurs at pH 8.2, of dehydropeptidase at pH 7.2, and of the effect of pyruvate on the desamidation of glutamine at pH 7.2. The maximum effect of the pyruvate occurs at a pH at which the desamidation of glutamine is almost nil.

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STUDIES ON THE EFFECT OF PYRUVATE ON THE DESAMIDATION OF GLUTAMINE, ASPARAGINE, AND RELATED COMPOUNDS [1]

By VINCENT E. PRICE, *assistant surgeon*, and JESSE P. GREENSTEIN, *principal biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The desamidation of glutamine and asparagine in aqueous extracts of rat liver is considerably increased by pyruvate at a pH (6.5–6.8) where the activity of glutaminase and asparaginase is minimal (*1*). The studies reported were performed after a single time interval of incubation and at a single extract concentration. The present studies are designed to show the time course of the reactions involved and have been extended to the study of peptides of glutamine and of asparagine; to an isomer of glutamine, namely, isoglutamine; to reactions occurring in kidney and other tissue extracts with these substances; and to an investigation of the effect of progressive dilution of rat liver extract on the desamidation of glutamine and of asparagine.

EXPERIMENTAL PROCEDURE

The aqueous extracts of rat liver and kidney were prepared as described (*1*). The digests consisted of 1 cc. of extract at concentrations designated, plus 1 cc. of amino acid amide or peptide amide at 0.014M concentration, plus either 1 cc. of water or 1 cc. of sodium pyruvate at 0.025M concentration. The ammonia evolved from the digests at the end of stated intervals was determined as before (*1*) and was corrected for the extract blanks which were run simultaneously.

Isoglutamine was prepared by the method of Bergmann and Zervas (*2*). Nitrogen value found was 19.0 percent, as compared with the theoretical value of 19.2 percent.

The synthesis of chloracetylasparagine and of glycylasparagine was described by Fischer and Koenigs (*3*). The preparation of chloracetylasparagine was modified and simplified, and a purer product obtained, by the following procedure. Chloracetylchloride and asparagine were coupled in dilute alkali in the proportions given by Fischer and Koenigs. After acidification of the reaction mixture with hydrochloric acid, the solution was extracted six times with ethyl acetate. The ethyl acetate extracts were combined, dried for 10 minutes over anhydrous sodium sulfate, and filtered through dry filter paper. On evaporation of the ethyl acetate solution in vacuo, the chloracetylasparagine crystallized out in white, glistening prisms. The product was filtered off, washed with dry ether, and dried. The yield of already pure chloracetylasparagine was 2–3 gm. from 10 gm. of asparagine. The melting point was 162° C., as compared with that of 149° given by Fischer and Koenigs. The nitrogen value found was 13.1 percent, as compared with the theoretical value of 13.4 percent.

Chloracetylglutamine has not hitherto been synthesized. It was prepared by the usual technique of coupling 1 mole of the glutamine with 1.2 moles of chloracetylchloride in slightly alkaline solution at 5° C. On acidification with hydrochloric acid, and similar to the technique found successful with chloracetylasparagine, the reaction mixture was extracted six times with ethyl acetate. After drying the combined ethyl acetate solutions, as described herein, the solution was evaporated in vacuo at 30° to a colorless oil. The oil was taken up in a little methyl alcohol, and the chloracetylglutamine crystallized out by the careful addition of petroleum ether. The yield of dried material was 1.5 gm. from 5 gm. of glutamine. The melting point was 132° C. The nitrogen value found was 12.4 percent, as compared with the theoretical value of 12.5 percent.

The synthesis of glycylasparagine from chloracetylasparagine was conducted according to the procedure described by Fischer and Koenigs (*3*). The nitrogen value found was 21.9 percent, as compared with the theoretical value of 22.2 percent.

Stock solutions of the chloracetyl peptides were brought to a pH of 6.8 with sodium hydroxide before adding to the digests. Purified solid sodium pyruvate was employed.

The initial pH of all digests was 6.7–6.8. At the end of a 4-hour incubation period, the pH of the digests generally falls to 6.5–6.6.

RESULTS

The time course of the desamidation of glutamine alone and in the presence of added pyruvate in extracts of rat liver and kidney is shown in figure 1. The liver extract concentration was equivalent to 333 mg. of tissue per cubic centimeter, that of the kidney, 166 mg. of tissue per cubic centimeter. It is evident that in both tissues the desamidation of the glutamine is increased in the presence of pyruvate. The glutaminase activity of the kidney is much higher than that of the liver.

A similar study of the desamidation of asparagine is shown in figure 2. The concentrations of liver and of kidney extract are as given for figure

[1] Received for publication January 2, 1947.

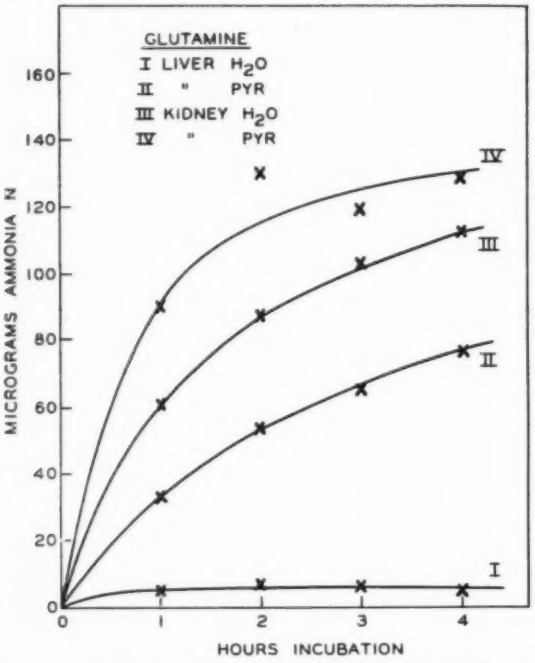

FIGURE 1.—Time course of desamidation of glutamine in rat liver and kidney extracts alone (H_2O) and in the presence of pyruvate (PYR).

1. The desamidation of asparagine is increased in the liver when pyruvate is added, but not in the kidney. No increase in desamidation of asparagine was noted after addition of pyruvate in kidney extracts when the concentration of these extracts was raised to that of the liver, namely, the equivalent of 333 mg. of tissue per cubic centimeter.

The asparaginase activity of liver is higher than that of glutaminase, but the reverse is true of the kidney.

The study of chloracetylglutamine (CAG) in the tissue extracts described is shown in figure 3. The extract concentrations are the same as those given for figure 1. Like glutamine, the desamidation of chloracetylglutamine in both liver and kidney extracts is increased when pyruvate is added. Also like glutamine, the desamidation of the peptide is greater in kidney than in liver.

The study of the desamidation of chloracetylasparagine (CAA) and of glycylasparagine (GA) is shown in figure 4. The extract concentrations are the same as those given for figure 1. At each time interval, the points on the curves are given for the desamidation of the substrates with and without the presence of added pyruvate. The upper points in each case refer to the values obtained in the presence of pyruvate. It is believed that the small differences shown in the few pairs of points are not significant, and the conclusion is that in neither liver nor kidney extracts is the desamidation of chloracetylasparagine or glycylasparagine increased in the presence of added pyruvate. It is of interest that the desamidation of glycylasparagine occurs more rapidly than does that of chloracetylasparagine.

The study of the desamidation of isoglutamine is shown in figure 5. The liver and kidney extract concentrations are the same as those given for figure 1. At every point, the desamidation values for the isoglutamine with or without pyruvate were nearly congruent, and the conclusion is reached that, unlike glutamine under identical conditions, the desamidation of isoglutamine is not affected by the presence of pyruvate. The desamidation of isoglutamine in both tissues is much greater than that of glutamine, and like the case of glutaminase, the isoglutaminase is much higher in kidney than in liver.

The effect of progressive dilution of the rat liver extract on the influence of pyruvate on the desamidation of glutamine and of asparagine is shown in figure 6. The augmenting effect of pyruvate on the desamidation of both amino acid amides is highest at the highest extract concentrations and progressively decreases with dilution

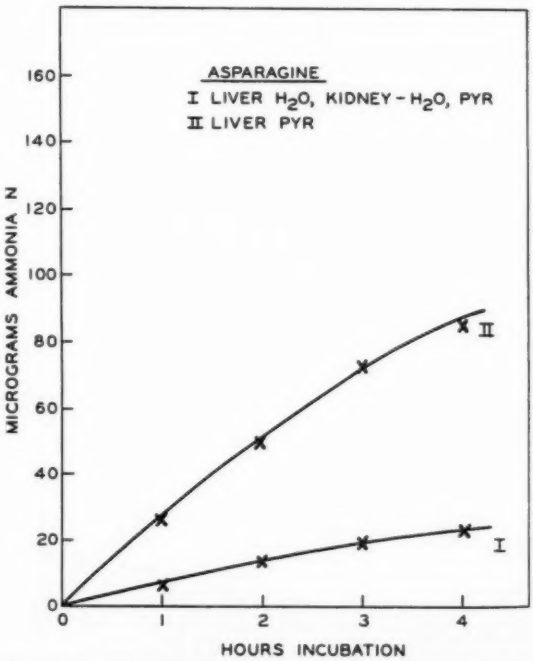

FIGURE 2.—Time course of desamidation of asparagine in rat liver and kidney extracts alone (H_2O) and in the presence of pyruvate (PYR).

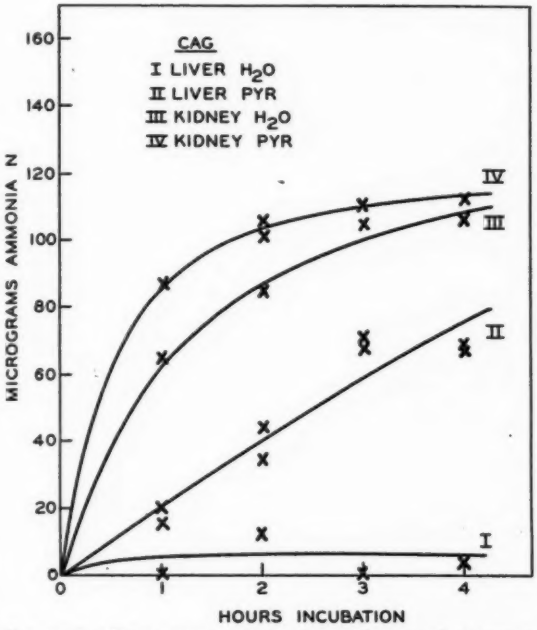

FIGURE 3.—Time course of the desamidation of chloracetylglutamine (CAG) in rat liver and kidney extracts alone (H_2O) and in the presence of pyruvate (PYR).

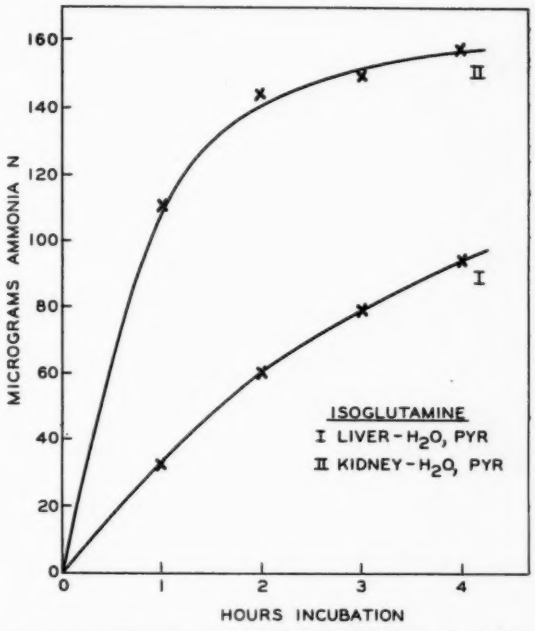

FIGURE 5.—Time course of the desamidation of isoglutamine in rat liver and kidney extracts, alone (H_2O) and in the presence of pyruvate (PYR).

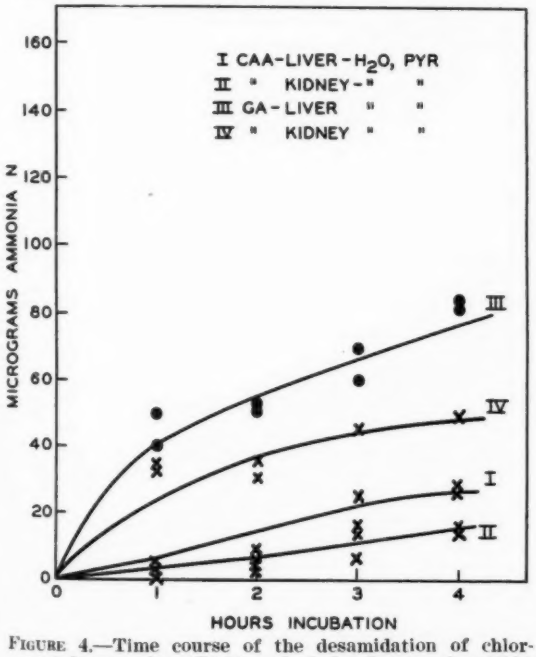

FIGURE 4.—Time course of the desamidation of chloracetylasparagine (CAA) and of glycylasparagine (GA) in rat liver and kidney extracts alone (H_2O) and in the presence of pyruvate (PYR).

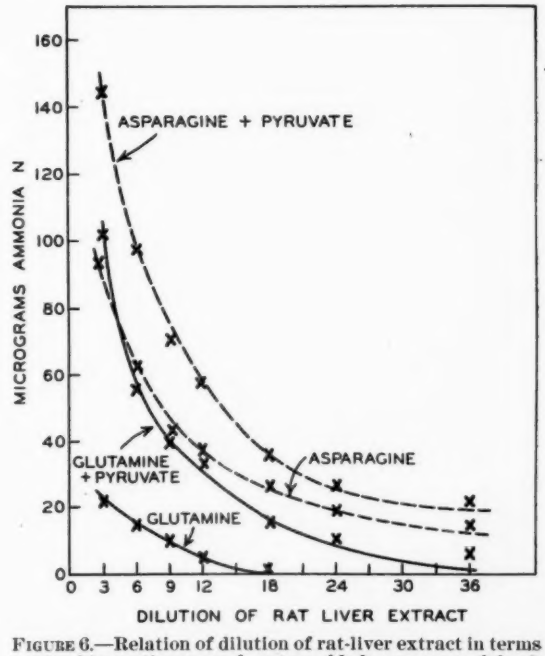

FIGURE 6.—Relation of dilution of rat-liver extract in terms of cubic centimeters of water added per gram of fresh liver tissue to the desamidation of glutamine and of asparagine.

of the extract. The desamidation of glutamine in the presence of pyruvate is appreciable, long after the glutaminase is diluted out.

The desamidation of glutamine and of asparagine in the presence and absence of added pyruvate in extracts of various rat and rabbit tissues is described in table 1.

TABLE 1.—*Desamidation of glutamine and asparagine in extracts of various tissues*[1]

Tissue	Ammonia nitrogen evolved from—			
	Glutamine	Glutamine and pyruvate	Asparagine	Asparagine and pyruvate
Rat:	*Micrograms*	*Micrograms*	*Micrograms*	*Micrograms*
Liver	[2] 10	98	[2] 39	152
Kidney	[2] 120	183	[2] 47	47
Spleen	27	32	11	15
Brain	20	40	0	9
Pancreas	45	47	15	15
Muscle	19	25	8	10
Rabbit:				
Liver	0	19	0	0
Kidney	74	74	5	5

[1] Digest composed of 1 cc. amide solution at 0.014M plus either 1 cc. pyruvate at 0.025M, plus 1 cc. aqueous tissue extract equivalent to 333 mg. tissue. All data corrected for tissue blanks. Digestion period 4 hours at 37° C. pH 6.8.
[2] Addition of 1 cc. 0.025M sodium acetate had no effect on the desamidation.

It appears that a significant effect of pyruvate in increasing the desamidation of glutamine is noted only in rat liver and kidney, and of asparagine only in rat liver. Many tissues appear to have appreciable glutaminase activity, i. e., rat pancreas and rabbit kidney, and appreciable asparaginase activity, i. e., rat kidney, but added pyruvate in extracts of these tissues has little or no effect in increasing the desamidation of the respective amino acid amides.

To emphasize further the effect of pyruvate, sodium acetate in the same concentration as pyruvate was added to the digests. No effect whatever of acetate on the desamidation of the amino acid amides was noted under conditions whereby pyruvate greatly increased their desamidation.

DISCUSSION

The results presented in this paper have confirmed and extended the earlier observations (*1*) on the increased desamidation of glutamine and of asparagine in the presence of added pyruvate in rat liver extracts. From table 1 it is clear that the effect is limited largely to the liver for both amides and to the kidney for glutamine only. There is no apparent relation between the pyruvate effect and the level of glutaminase or asparaginase activity, and indeed it is believed that the effect of pyruvate is independent of the activity of these two enzymes and proceeds by another mechanism. This mechanism is presumed to involve the condensation of glutamine with pyruvate to form a dehydropeptide, the peptide bond of which involves the γ carbon atom of glutamic acid, followed by the splitting of the dehydropeptide to form glutamic acid, ammonia, and pyruvate (*1*, cf. also *4*). Whether this mechanism as postulated is true or not, it is evidently possible for a tissue to have a high glutaminase or asparaginase activity with or without a pyruvate effect; and it is also possible for a tissue to have a low glutaminase or asparaginase activity with or without a pyruvate effect (table 1). In the case of tissues possessing high glutaminase or asparaginase activity, it is obviously necessary when studying the augmenting effect of pyruvate on desamidation to choose such experimental conditions that the pyruvate effect will be evident; otherwise the glutamine and asparagine will be desamidated by the glutaminase and asparaginase before the pyruvate has an opportunity to exert its effect.

The fact that the desamidation of isoglutamine in liver and in kidney extracts is unaffected by added pyruvate suggests that the effect of pyruvate is limited to amino acid amides with amide groups on either β or γ carbons, and not on the α carbon. This is supported by the observation that chloracetamide and benzoylarginineamide are not affected by addition of pyruvate to rat liver digests of these substances.[2]

The study of the chloracetyl peptides of glutamine and of asparagine was chosen because in these compounds the α amino group of these amino acid amides is substituted by the chloracetyl radical. The carboxypeptidase activity of liver is extremely weak or nonexistent (*5*), and it is therefore not expected that these peptides are split at the peptide bond during the incubation period. Any ammonia that appears in the digests of these peptides must therefore have come from the desamidation of their amide groups. The fact that the desamidation of chloracetylglutamine is increased by pyruvate (fig. 3) lends weight to the condensation theory proposed (*1*). The fact, however, that the rate of desamidation of chloracetylglutamine is less than that of glutamine, and that the pyruvate effect is nonexistent with both chloracetylasparagine and glycylasparagine suggests that the presence of a free α amino group in glutamine increases the efficiency of the pyruvate effect and that the presence of a free α amino group in asparagine is essential for the pyruvate effect.

The powerful asparaginase activity of rat liver as compared with glutaminase activity is shown in figure 6. The augmenting effect of pyruvate on the desamidation of both glutamine and asparagine falls off with progressive dilution of the liver extract. In the case of glutamine, the augmenting effect of pyruvate extends beyond the dilution at which glutaminase activity drops out and is appreciable beyond 12 times the dilution of the extract (fig. 6).

[2] Unpublished observations.

SUMMARY

The time course of the desamidation of glutamine, isoglutamine, asparagine, chloracetylglutamine, chloracetylasparagine, and glycylasparagine in the presence and absence of pyruvate was studied in extracts of rat liver and kidney. Pyruvate increased the desamidation of glutamine and chloracetylglutamine in both liver and kidney extracts, and of asparagine only in liver extracts. Pyruvate had no effect on the desamidation in either tissue of isoglutamine, chloracetylasparagine, or glycylasparagine.

Extracts of spleen, brain, muscle, and pancreas showed no significant pyruvate effect with either glutamine or asparagine.

The effect of pyruvate on the desamidation of glutamine of asparagine fell off with dilution of liver extract.

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DESAMIDATION OF AMINO ACID AMIDES IN RAT-LIVER EXTRACTS OF VARYING CONCENTRATIONS[1]

By JOSE M. GONÇALVES,[2] *fellow of the Rockefeller Foundation*, VINCENT E. PRICE, *assistant surgeon*, and JESSE P. GREENSTEIN, *principal biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

It has been shown that digests of glycyldehydroalanine with glutamine in the presence of concentrated rat-liver aqueous extracts (three to six times the weight of water per unit weight fresh tissue) yield values of ammonia which are higher than the sum of the ammonia values yielded in digests of glycyldehydroalanine and of glutamine considered separately (*1*). This has been interpreted as being due to the presence of pyruvate in the peptide-amide mixtures, the pyruvate being furnished by the action of dehydropeptidase I on the glycyldehydroalanine (*2*), and producing an increase in desamidation of the glutamine (*1, 3*).

The present study extends the investigation to digests of other amides with glycyldehydroalanine in the presence of varying concentrations of rat-liver aqueous extracts.

MATERIALS AND METHODS

The methods of preparing the rat-liver extracts for the enzymatic determinations, the determinations of evolved ammonia from the digests, and the preparation and properties of the substrates have been described (*1, 4*). The ammonia values in digests containing the substrates are corrected for the extract blanks. None of the substrates by themselves yield ammonia in the course of the reactions, i. e., the substrate blanks are negative. The digests for ammonia determinations contained 1 cc. of fresh aqueous, rat-liver extract, 1 cc. veronal acetate buffer at pH 6.8, either 1 cc. of water or 1 cc. of glycyldehydroalanine at 0.025M concentration, and either 1 cc. of water or 1 cc. amide at 0.014M concentration. The period of incubation was 4 hours at 37° C.

RESULTS

Figures 1–5 refer to studies of the ammonia evolved from rat-liver digests containing extracts at various dilutions with various amides. The abscissa in each figure represents the cubic centimeters of water added per gram of fresh homogenized tissue.

It is evident by inspection of figures 1–5, that of all the amides studied, only glutamine, asparagine, and chloracetylglutamine are desamidated to a greater extent in the presence of glycyldehydro-

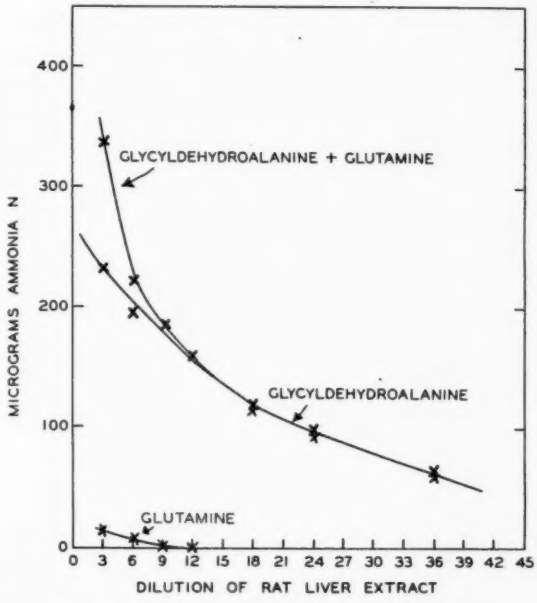

FIGURE 1.—Ammonia evolved from rat-liver digests at various extract concentrations with glycyldehydroalanine alone, glutamine alone, and glycyldehydroalanine and glutamine together.

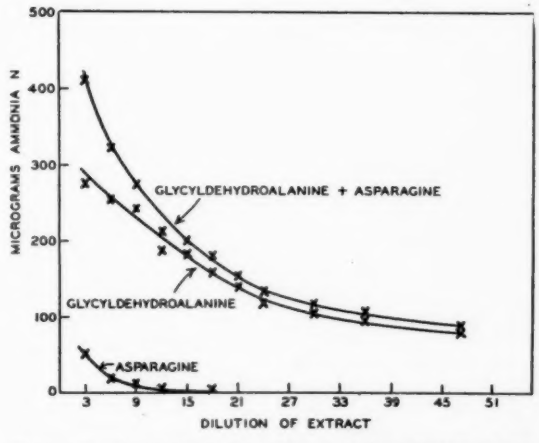

FIGURE 2.—Ammonia evolved from rat-liver digests at various extract concentrations with glycyldehydroalanine alone, asparagine alone, and glycyldehydroalanine and asparagine together.

[1] Received for publication January 6, 1947.
[2] On leave from the Department of Biophysics, University of Brazil, Rio de Janeiro.

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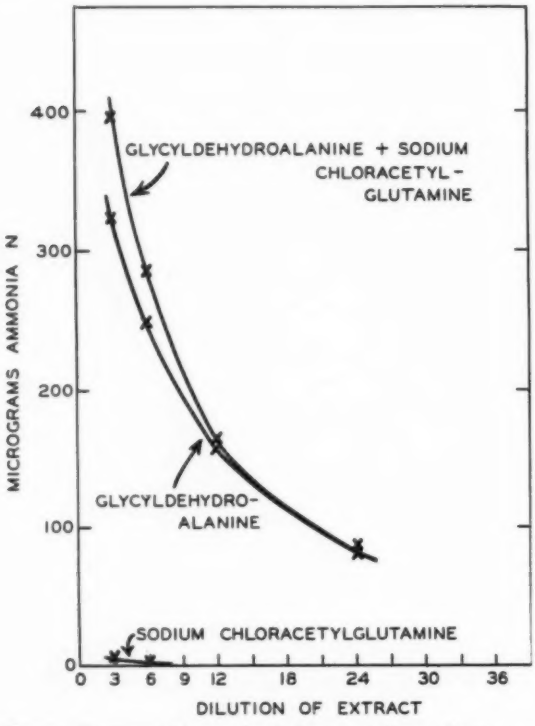

FIGURE 3.—Ammonia evolved from rat-liver digests at various extract concentrations with glycyldehydroalanine alone, chloracetylglutamine alone, and glycyldehydroalanine and chloracetylglutamine together.

alanine than in its absence. This is presumably due to the effect of the pyruvate formed as a result of the degradation of the peptide by the dehydropeptidase present in the extract and is consistent with the results obtained on the effect of added, pure sodium pyruvate on the desamidation of these amides (*3*). The augmenting effect on glutamine, asparagine, and chloracetylglutamine exists only at the higher extract concentrations (figs. 1, 2, and 3) (*1*). At the lower extract concentrations of these three amides, the curves for mixtures of glycyldehydroalanine and either glutamine, asparagine, or chloracetylglutamine, coincide with that of glycyldehydroalanine alone. Isoglutamine and chloracetylasparagine show no effect at any extract concentration (figs. 4 and 5) (*4*), the curves for the sum of the ammonia values of peptide and amide taken separately being congruent throughout its length with the corresponding curve for the ammonia values of mixtures of peptide and amide.

A composite chart of the ammonia data obtained with all of the amides studied is given in figure 6.

In the course of these investigations we have employed three different samples of glutamine.[3] In concentrated extracts of rat liver, and in the presence and in the absence of glycyldehydroalanine or of added sodium pyruvate all three samples of glutamine behaved identically, i. e., the ammonia values for each of the three preparations under identical experimental conditions were very nearly the same. However, in digests of mixtures of glycyldehydroalanine with glutamine in dilute rat-liver extracts (12–48 times the weight of water per unit weight fresh tissue), the Lederle glutamine caused a marked inhibition of 40–60 percent in the splitting of glycyldehydroalanine which was revealed both by ammonia determinations and by the spectrophotometric method of assaying dehydropeptidase activity (*5*). This inhibition could not be overcome by the addition of as much as 4 mg. of glutathione to the reaction mixture, nor could it be removed by recrystallization of the product, the inhibitory effect per milligram of

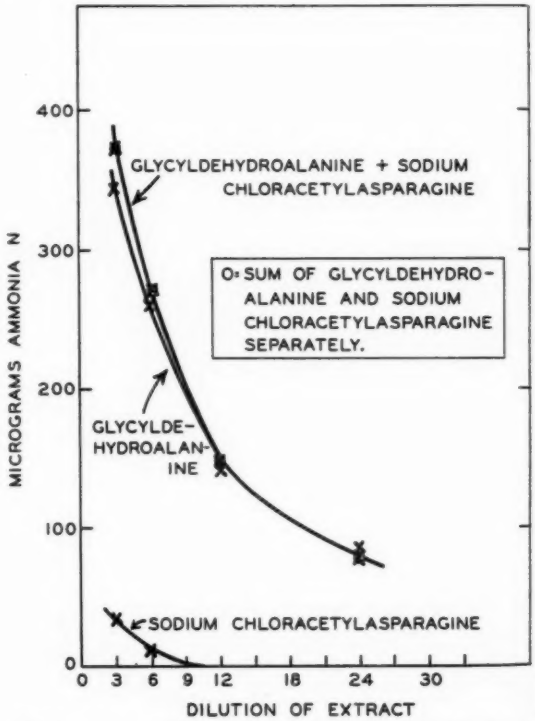

FIGURE 4.—Ammonia evolved from rat-liver digests at various extract concentrations with glycyldehydroalanine alone, chloracetylasparagine alone, and glycyldehydroalanine and chloracetylasparagine together.

[3] Generously furnished, respectively, by Dr. G. W. Pucher, of the Connecticut State Agricultural Experiment Station, Dr. Joseph S. Fruton, of Yale University, and the Lederle Laboratories of the American Cyanamide Co.

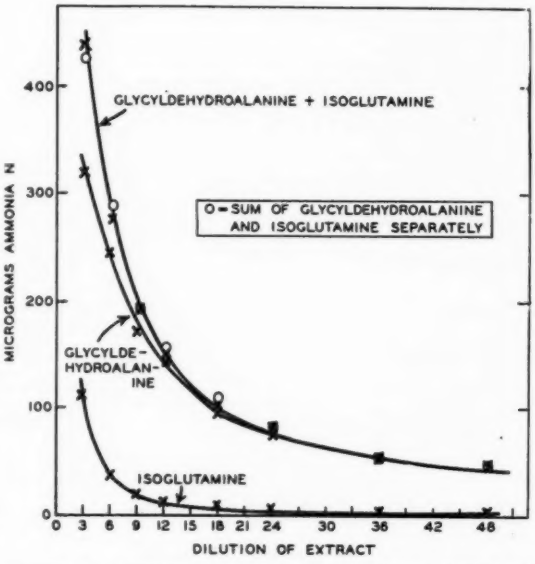

FIGURE 5.—Ammonia evolved from rat-liver digests at various extract concentrations with glycyldehydroalanine alone, isoglutamine alone, and glycyldehydroalanine and isoglutamine together.

glutamine being identical for the recrystallized material and for the mother liquor. Since the experiments with glutathione seemed to rule out the possibility of contamination by heavy metals, there remained the possibility of a slight contamination of the product by arginine. This, however,

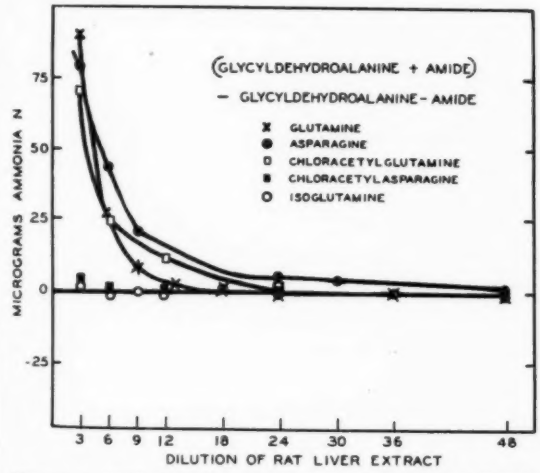

FIGURE 6.—Differences in ammonia values between digests containing glycyldehydroalanine plus amide and the sum of glycyldehydroalanine and of the amide taken separately, as a function of the dilution of rat-liver extract.

was ruled out by the failure of arginine when added to digests containing glycyldehydroalanine to affect the splitting of the dehydropeptide. The Lederle product is pure from the analytical standpoint, the material giving 90–96 percent of the theoretical yield of amide nitrogen. The samples of glutamine furnished by Drs. Pucher and Fruton produced no such inhibition of dehydropeptidase activity in dilute tissue extracts, and the data on glutamine, reported in this paper, have been obtained with Dr. Pucher's material.

DISCUSSION

The enzymatic degradation of glycyldehydroalanine over a wide range of liver-extract concentrations leads to the production of glycine, pyruvate, and ammonia (*6*). In relatively concentrated liver extracts, whether in the presence of pyruvate added as the pure sodium salt, or in the presence of pyruvate furnished by the concomitant degradation of glycyldehydroalanine, the desamidation of glutamine, of asparagine, and of chloracetylglutamine is considerably increased. That this increase in ammonia production is not due to an increase in dehydropeptidase activity is shown by the fact that with glutamine at least, the increase in ammonia can be accounted for by a corresponding decrease in the amide nitrogen of the amino acid amide (*1*, *3*). With progressive dilution of the tissue extract, the effect on the amides diminishes and finally disappears. Chloracetylasparagine and isoglutamine are not desamidated to an increased extent in the presence of pyruvate, whether added as the pure sodium salt (*4*) or whether furnished by the splitting of glycyldehydroalanine (figs. 4 and 5). None of the amides reported in this paper affect the splitting of the dehydropeptide.

The lack of effect of pyruvate on the desamidation of isoglutamine in contrast with the strong effect of this keto acid on glutamine, together with the lack of effect of pyruvate on the desamidation of benzoylarginine amide [4] suggests that the reaction between pyruvate and amides may be confined to those amides on the side chains of the amino acids and is not concerned with amides on the α carbon atom. Among natural products, only glutamine and asparagine are representative of the ω carboxylic groups on amino acids. The difference in reactivity of chloracetylglutamine and of chloracetylasparagine toward pyruvate suggests that in the interaction of glutamine with pyruvate the presence of an α amino group is not necessary, whereas in the interaction of asparagine with pyruvate the presence of the α amino group is necessary. Although the desamidation of glutamine and of asparagine is increased in

[4] Greenstein, J. P., and Carter, C. E.: Unpublished data.

the presence of pyruvate, the mechanism whereby this effect is achieved need not necessarily be identical in all respects for the two amides.

SUMMARY

The activity of dehydropeptidase I with glycyldehydroalanine as substrate was studied in rat-liver digests at various dilutions of tissue extracts, in the presence of glutamine, isoglutamine, chloracetylglutamine, asparagine, and chloracetylasparagine. At high extract concentrations, the pyruvate derived by enzymatic break-down of the peptide resulted in an increased desamidation of glutamine, chloracetylglutamine, and asparagine; the desamidation of isoglutamine and of chloracetylasparagine was not affected. At lower extract concentrations, the curves relating ammonia production to dilution of extract are congruent for glycyldehydroalanine plus amide and for glycyldehydroalanine alone.

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DESAMIDATION OF GLUTAMINE AND ASPARAGINE IN NORMAL AND NEOPLASTIC HEPATIC TISSUES [1]

By MAURICE ERRERA,[2] *research fellow*, and JESSE P. GREENSTEIN, *principal biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Aqueous extracts of normal, adult, rat liver desamidate glutamine and asparagine relatively slowly. In the presence of added pyruvate, the desamidation of these amides is very considerably increased (*1–4*). The pyruvate is not consumed in the reaction. It has been assumed that the pyruvate forms an intermediate compound with the amide, of the nature of a dehydropeptide, which is subsequently split to yield products which include ammonia and the regenerated keto acid.

It was considered of interest to study the desamidation of glutamine and asparagine and the effect of pyruvate thereon in aqueous extracts of such homologous hepatic tissues as regenerating, fetal, and neoplastic liver, and to compare the behavior of these tissues with that of normal, resting, adult liver.

EXPERIMENTAL PROCEDURE

The tissues were removed from the freshly killed animals and were ground to a paste with a little sand in a glass mortar. The homogenate was then taken up in the designated amount of distilled water, lightly centrifuged, and the supernatant used as the source of enzyme.

Regenerating rat liver was studied 48 hours after lobectomy. Fetal rat livers were obtained from animals which were about 2 weeks pregnant. The primary rat hepatomas had been induced by the feeding of *p*-dimethylaminoazobenzene and were donated by Dr. J. White. The transplanted mouse hepatomas, 98/15 in C3H mice, and 587 in A mice, were donated by Dr. A. B. Eschenbrenner, and were in the thirty-first and fifty-sixth transplant generation, respectively. These hepatomas arose spontaneously originally in mice of these strains. Necrotic areas were removed from all tumors studied.

The digests consisted of 1 cc. of extract, plus 1 cc. of veronal acetate buffer at pH 6.8, plus either 1 cc. of water or 1 cc. of amide at 0.014M concentration, plus either 1 cc. of water or 1 cc. of sodium pyruvate at 0.025M concentration. Serial dilutions of the extract were performed by adding water to the stock extract prior to setting up the digests. The desamidation of the amides was followed by measuring the ammonia evolved according to the method described (*1*). All digests were corrected for the tissue blanks. The incubation period was 4 hours at 37° C. Beginning and final pH of the digests did not vary from 6.7–6.8. The data were obtained on six samples of each kind of adult tissue, and were uniformly consistent. The data on the fetal livers were obtained on three samples of pooled tissues. In all cases, the variation in readings on replicate determinations was ± 5 percent, and between various samples was ± 15 percent.

RESULTS

Figure 1 describes the results obtained with regenerating rat liver. To all intents, the data are practically identical with those obtained under similar conditions with normal, resting adult liver (*1–4*), i. e., the desamidation of asparagine is notably greater than that of glutamine, while in the presence of added pyruvate the desamidation of

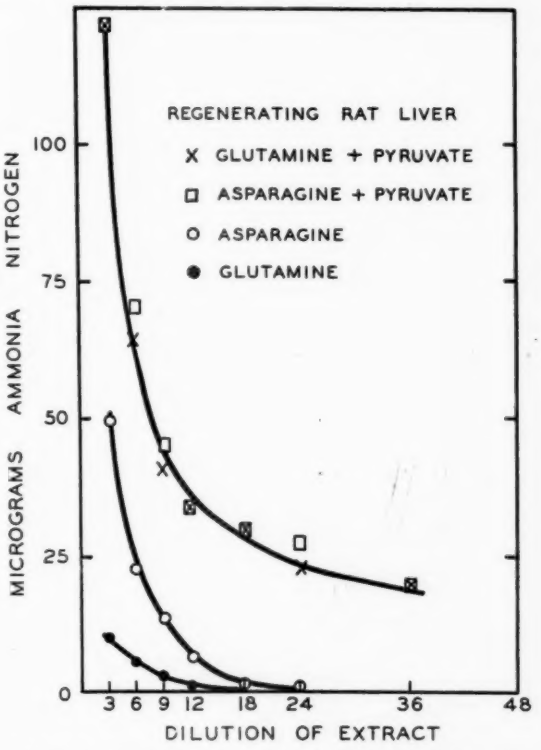

FIGURE 1.—The desamidation of glutamine and of asparagine in the presence and in the absence of added pyruvate as a function of the extract concentration. Abscissa refers to cubic centimeters water added per gram of regenerating rat-liver tissue.

[1] Received for publication January 20, 1947.
[2] On leave from University of Brussels, Belgium.

both amides is enormously increased and occurs at nearly the same extent.

Figures 2 and 3 relating, respectively, to the primary rat hepatoma and to rat fetal liver are considered together because of their similarity. In both tissues, and in contrast with adult liver, there is very little capacity to desamidate asparagine, whereas there is an appreciable capacity to desamidate glutamine. In the presence of added pyruvate, the desamidation of both amides is increased, the desamidation of glutamine occurring to a greater extent than that of asparagine. On the basis of total weight of tissue taken for the extracts, the extent of desamidation of glutamine in fetal liver and in the hepatoma is considerably greater than in resting or regenerating adult liver, that of asparagine is considerably less; in the former two tissues, the desamidation of the amides

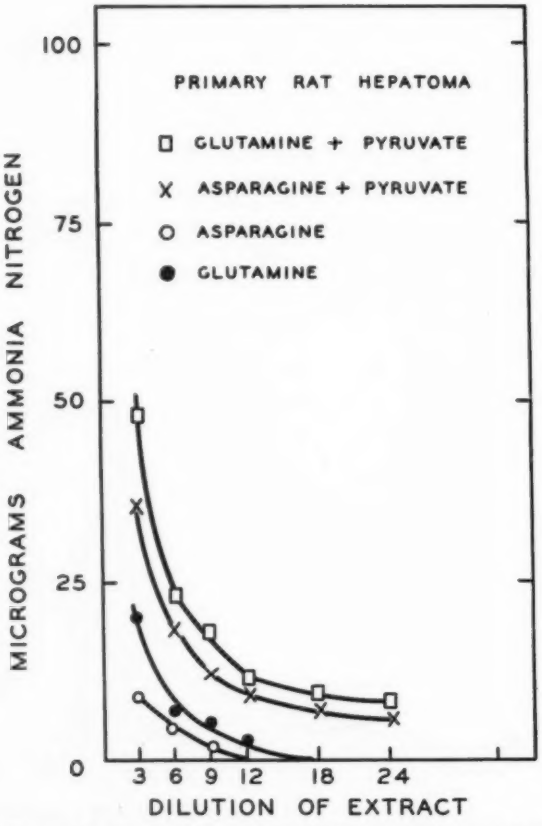

FIGURE 2.—Desamidation of glutamine and of asparagine in the presence and in the absence of added pyruvate as a function of the extract concentration. Abscissa refers to cubic centimeters of water added per gram of primary-rat-hepatoma tissue.

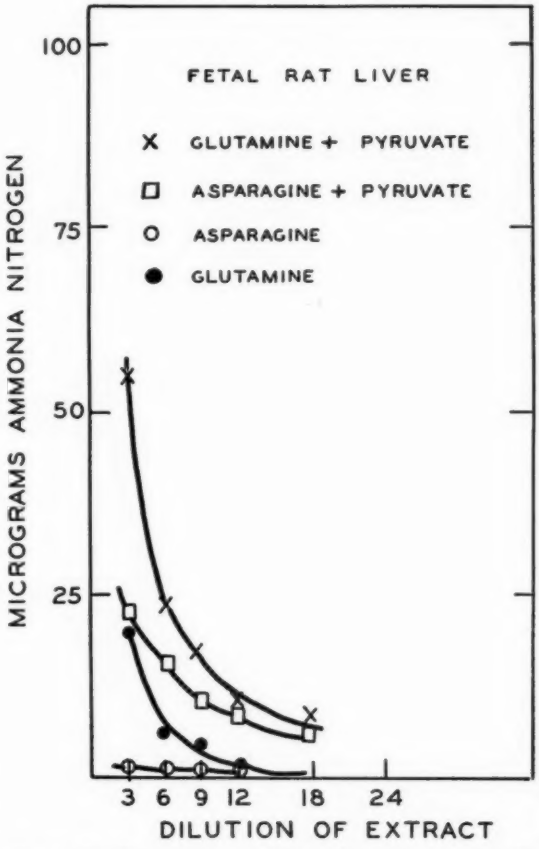

FIGURE 3.—Desamidation of glutamine and of asparagine in the presence and in the absence of added pyruvate as a function of the extract concentration. Abscissa refers to cubic centimeters of water added per gram of fetal-rat-liver tissue.

in the presence of pyruvate is much less than in the latter two tissues.

For equal weights of tissue taken, the total extractable nitrogen per unit volume in extracts of resting and regenerating adult liver is roughly twice that of fetal and neoplastic liver. On the basis of total nitrogen per cubic centimeter of extract, therefore, the glutaminase activity of fetal and neoplastic liver is even still greater than of resting and regenerating adult liver; asparaginase activity is still less, while the extent of desamidation of both amides in the presence of pyruvate is not far from being of the same order of magnitude in all four tissues.

Figure 4 gives the data obtained on the transplanted hepatoma 98/15 in C3H mice and on the normal adult livers of C3H mice. Essentially similar results were obtained on hepatoma 587 in

A mice. The following points may be noted: (1) The asparaginase activity of mouse liver is very much higher than that of glutaminase in this tissue, and is also much greater than the asparaginase activity of rat liver; (2) the glutaminase activity of mouse liver and of rat liver is very nearly the same; (3) as in rat liver, the addition of pyruvate to extracts of mouse liver results in a considerable increase in the desamidation of glutamine, the increase in the desamidation of asparagine being less marked because of the originally high asparaginase activity of the mouse liver; (4) as in rat liver, the desamidation of the two amides in mouse liver extracts containing added pyruvate is very nearly the same; and (5) as in rat hepatoma (and in rat fetal liver), the asparaginase activity of the mouse hepatoma is almost nil, the glutaminase activity of the mouse hepatoma is appreciable, while the desamidation of both amides is increased, that of glutamine more than that of asparagine, by the addition of pyruvate.

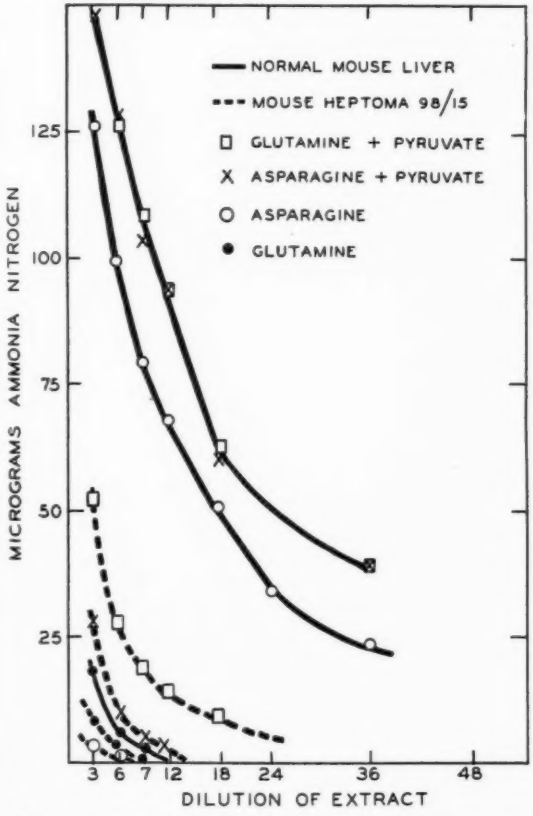

FIGURE 4.—Desamidation of glutamine and of asparagine in the presence and in the absence of added pyruvate as a function of the extract concentration. Abscissa refers to cubic centimeters of water added per gram of mouse liver or mouse hepatoma tissue.

Other transplanted mouse tumors studied included the fibrosarcoma CR180, a lymphosarcoma in dba mice, a mammary-gland carcinoma in C57 brown mice, and a squamous-cell carcinoma. Aqueous extracts of the three tumors last mentioned revealed little or no glutaminase or asparaginase activity, and no capacity to increase the desamidation of these amides in the presence of added pyruvate. The fibrosarcoma CR180 revealed a definite glutaminase activity, a still greater asparaginase activity, but no capacity whatever to increase the desamidation of these amides in the presence of added pyruvate.

DISCUSSION

Fetal liver apparently possesses little or no asparaginase activity but does possess a well-marked glutaminase activity. In the adult animal, the relative activity of these enzymes is reversed, asparaginase activity being much higher than that of glutaminase. When the adult liver becomes neoplastic, the fetal pattern is noted in the hepatoma, i. e., near disappearance of asparaginase and concomitant rise in glutaminase activity. That a tumor may possess the metabolic characteristics of the embryonic form is by now not surprising (*5–7*). The enzymatic activity of regenerating liver is practically identical with that of resting, adult liver, which is consistent with all earlier observations on the comparison of these two tissues (*6*).

The results obtained on the comparison of mouse liver and mouse hepatoma are consistent with those obtained on the comparison of rat liver and rat hepatoma. In both instances, liver has high asparaginase and low glutaminase, whereas hepatoma has a relatively high glutaminase and a very low asparaginase activity. Of the particular metabolic systems studied and reported in this paper, these are the distinguishing characteristics of the two kinds of hepatic tissue, i. e., normal and neoplastic. The desamidation of glutamine and of asparagine is considerably increased by added pyruvate in all four kinds of hepatic tissue studied, resting adult liver, regenerating liver, fetal liver, and hepatoma, but this is evidently because these tissues are hepatic in nature or in origin. It has been noted that the capacity of increasing the desamidation of glutamine and of asparagine by added pyruvate is, of all tissues studied, nearly an exclusive property of the liver (*3*). Extracts of rat kidney plus added pyruvate increase the desamidation of glutamine but not of asparagine, while extracts of spleen, brain, muscle, and pancreas treated with pyruvate do not affect the desamidation of either amide.

As noted previously, none of the other mouse tumors possess the capacity of increasing the desamidation of the amides in the presence of added pyruvate. It is therefore interesting to observe that this pyruvate effect is present in all the homologous hepatic tissues studied, is present in the fetal liver, is preserved when the fetal form develops into the adult liver, and is, furthermore, preserved when the adult liver is transformed into the neoplasm. In this respect, the neoplasm bears the imprint of the tissue of its origin. Such a preservation of a unique property of the normal tissue is relatively rare. When a normal tissue possesses a property which sets it off from other normal tissues, the tendency is to lose or sharply diminish the extent of this property when the tissue becomes neoplastic (*7*). The possession by a tumor of unknown etiology of the pyruvate effect on the desamidation of glutamine and asparagine might serve therefore to distinguish it as a hepatoma.

SUMMARY

The glutaminase and the asparaginase activity of normal, resting adult liver, of regenerating liver, of fetal liver, and of primary and transplanted hepatomas was determined, as was the effect of added pyruvate on the extent of desamidation of these amino acid amides.

Extracts of fetal liver and of the hepatoma possess appreciable glutaminase activity, and very little asparaginase activity. Extracts of resting adult liver and of regenerating liver possess very little glutaminase activity but have high asparaginase activity. The desamidation of both amides is considerably increased in all four kinds of homologous hepatic tissues by the addition of pyruvate. The possession of this pyruvate effect is a nearly unique characteristic of hepatic tissues, whether normal or neoplastic.

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MAST-CELL NEOPLASIA IN MICE [1]

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INTRODUCTION

Reports on the autonomous growth of cells characterized by basophilic granules are infrequent. These few reports, however, must be divided into two groups, since it is now generally agreed that the basophilic leukocyte and the tissue mast cell are distinct cellular entities (Michels (*1*), Doan and Reinhart (*2*)).

Leukemias in which the basophilic leukocyte had undergone a neoplastic change in man have been described by Doan and Reinhart. These authors believe that their three reported cases "may be the first recorded instances of primary, progressive, specific hyperplasia of basophil granule leucocytes," and that previous reports of supposed mast-cell leukemia in man can well have been wrongly diagnosed, since the basophilic leukocytes are frequently increased in number during the course of chronic myelogenous leukemia. Lillie (*3*) has reported a case of mast-myelocyte leukemia in a cat. This case was characterized by an extensive mast-myelocyte infiltration in the splenic pulp, mast-myelocyte nodules in the liver, and numbers of mast myelocytes in the hepatic and renal veins. The blood, lymph nodes, and bone marrow were not examined, since the nature of the process was not suspected when the autopsy was made. (The disease in cats may be less rare than in other species, since Bloom (*4*) states that he has seen several.)

True tumors of the tissue mast cells, or mastocytomas, are of doubtful occurrence in man, although urticaria pigmentosa may reveal considerable local accumulations of these cells. True mast-cell tumors, however, appear to be relatively frequent in the dog, and Bloom (*4*) has reported five cases developing in the skin, two of which involved other organs and were considered to indicate either metastatic or multicentric origin. Sabrazès and Lafon (*5*) have reported a tumor the size of an orange, found on the lip of a horse and consisting chiefly of tissue mast cells with massive infiltration of eosinophilic leukocytes, and frequent hemorrhagic foci.

An increase in mast cells in the skin of the mouse after exposure to tar or methylcholanthrene has been reported by a number of authors, among them de Vinyals (*6*), Twort and Twort (*7*), and Cramer and Simpson (*8*). The last 2 authors have recently reviewed the literature on mast cells in experimental skin carcinogenesis and have reported their own observations following the application of methylcholanthrene. The accumulations which they describe appear to have remained localized, however, and only two reports have been found of a probable neoplastic change in these cells in the mouse. Twort and Twort observed a diffuse infiltrative condition of mast cells in the internal organs of 1 mouse following the application of tar. This condition seemed to have been the cause of death. Lignac (*9*) has reported 2 additional cases with mast-cell infiltration. These he considered a special form of leukemia, in which the characteristic cell contained basophilic granules. He found these 2 cases among 8 mice which had developed "benzol leukemia." These appeared after 54 mice had been repeatedly exposed to this agent.

At this point the question may properly be raised as to the correct terminology of mast-cell neoplasia in the mouse. The frequently encountered neoplasias of the mouse which appear to originate in lymphoid cells and are characterized by a generalized involvement of the lymphoid organs and infiltrations at other sites are referred to by Richter and MacDowell (*10*) as leukemia, even when no neoplastic cells are detected in the peripheral blood. In these cases of mast-cell neoplasia the cells have the distribution commonly found in this process in the mouse, yet the cells are almost certainly derived from tissue mast cells, and not from blood or bone marrow, since it is doubtful that a true mast leukocyte occurs in this species. Jaffé (*11*) states that basophilic leukocytes are never found in the blood of normal mice, and reports to be published from the National Cancer Institute on studies of the peripheral blood of mice indicate that they are extremely rare. Lignac (*9*) reports a basophilia of 4 percent in one of his cases and considers this evidence of a true leukemia. In the general problem of mouse leukemia, it is hoped that cases of mast-cell neoplasia will be helpful in determining the distribution and observing the origin and spread of the neoplastic growth because of the metachromatic staining which this cell takes.

CASE REPORTS

During the examination of histopathologic material from mice of several groups of an experiment [2] designed to study the incidence of renal disease and amyloidosis, five cases of mast-cell neoplasia were discovered.

A characteristic type of renal disease having a morphologic resemblance to pyelonephritis occurs in almost 100 percent of the strain A mice living to 18 months of age, whereas it occurs only rarely

[1] Received for publication February 4, 1947.

[2] Heston, W. E., and Deringer, M. K.: Unpublished data.

in strain L mice of the same age. Andervont (*12*) noted the occurrence of "cystic degeneration of the kidneys" in the majority of strain A mice at 18 months of age, and Gorer (*13*) reported the occurrence of a cystic disease of the kidneys in approximately 100 percent of strain A mice over 16 months of age. The experiment from which the cases described herein were taken was designed to determine the incidence of this type of renal disease in strain A, strain L, their reciprocal first-generation hybrids, ALF_1 and LAF_1, the second-generation hybrids, ALF_2 and LAF_2, and the two backcrosses, LA-ABC and LA-LBC. Groups of animals were scheduled for autopsy at 18 and 24 months of age, and pieces of tissue from kidney, duodenum, and spleen were taken routinely from each animal. Lesions from any other organ or tissue were also fixed.

A total of 861 mice, distributed among the various groups as follows, has been autopsied: strain A, 84; strain L, 167; ALF_1, 85; LAF_1, 139; ALF_2, 90; LAF_2, 104; LA-ABC, 111; and LA-LBC, 81. Three of the five cases of mast-cell neoplasia which were found occurred in ALF_1 hybrid mice at 20, 22, and 24 months of age (table 1). One case was found in a LAF_1 hybrid at 24 months of age, and 1 in a backcross animal, LA-ABC, at 18 months of age. It is possible that cases of mast-cell neoplasia might have occurred in the strain A mice had they lived beyond 18 months of age. It is regrettable that so little microscopic material from each case was available for study, but the condition was not suspected at autopsy, and there was usually nothing on gross observation to indicate a pathologic process. Consequently only those tissues were taken which were pertinent to the problem of renal disease and amyloidosis, except in case 1, where hemorrhagic lesions appeared in the lungs, and case 4, where an enlarged lymph node was seen. Table 2 lists the organs examined in each case. Case 1 is described in most detail, and the other 4 cases are described with this as a reference. All tissues were fixed in Tellyesniczky's fluid, consisting of 2 parts formol, 20 parts 70 percent alcohol, and 1 part acetic acid. The stained sections indicated that in this alcoholic solution most of the mast-cell granules were preserved. A hematoxylin-eosin stain was made routinely after paraffin embedding. When mast cells were recognized as especially numerous in sections from any autopsy after this procedure, toluidine blue stains were applied, and the prussian-blue reaction for iron with a fuchsin counterstain was also used.

TABLE 1.—*Occurrence of mast-cell neoplasia in different groups of mice*

[Numerator = mice developing mast-cell neoplasia. Denominator = mice dying without mast-cell neoplasia]

Group	Number of mice	Time in months																		Total	Incidence of mast-cell neoplasia
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
																					Pct.
A	84	.....	.....	.....	0/1	0/1	0/3	0/19	0/12	0/4	0/3	0/7	0/34	.....	.....	.....	.....	.....	.....	.....	0/84	0
L	167	.....	.....	.....	0/3	0/1	0/1	0/1	0/2	0/6	0/8	0/7	0/84	0/3	0/4	0/12	0/6	0/3	0/25	0/1	0/167	0
LAF_1	139	.....	.....	.....	.....	.....	0/1	.....	.....	.....	.....	0/1	0/42	0/1	0/2	0/4	0/3	0/7	1/77	.....	1/139	.007
LAF_2	104	.....	.....	.....	0/1	0/2	0/6	0/5	0/5	0/1	0/3	0/2	0/45	0/1	0/2	0/6	0/6	0/1	0/18	.....	0/104	0
ALF_1	85	.....	.....	0/1	.....	.....	0/3	0/1	.....	0/2	.....	0/1	0/35	0/1	1/3	0/2	1/2	0/3	1/31	.....	3/85	.035
ALF_2	90	.....	0/1	0/1	.....	0/1	0/2	.....	0/1	0/1	0/8	0/1	0/44	0/2	0/5	.....	0/2	0/3	0/18	.....	0/90	0
LA-ABC	111	0/1	0/2	.....	.....	0/2	0/3	0/1	.....	0/2	0/3	0/5	1/56	0/1	0/4	0/1	0/6	0/2	0/22	.....	1/111	.009
LA-LBC	81	0/1	.....	0/3	.....	0/3	0/6	0/1	0/2	0/6	0/1	0/1	0/28	.....	0/4	0/7	0/2	0/2	0/14	.....	0/81	0

TABLE 2.—*Distribution of neoplastic mast cells*

No.	Type	Sex	Age	Spleen	Kidney	Duodenum	Lung	Adrenal	Pancreas	Vessels	Other organs
			Mos.								
1	ALF_1	M	20	+++	++	–	+++	(?)	.....	+Spleen..	
2	LAF_1	F	24	++	+	(?)	+	–	–	+Kidney..	
3	ALF_1	F	22	–	–	–	–	.....	–	–	+++Inguinal node.
4	LA-ABC	M	18	(?)	+++	+++	.....	.....	–	–	+Liver.
5	ALF_1	M	24	++	–	+	–	–	.....	–	

CASE 1

ALF_1 male, 20 months of age at autopsy revealed the presence of renal disease, multiple hemorrhages of lung, and enlargement of the spleen.

Spleen.—The largest number of mast cells were found in this organ (fig. 1). They were found principally within the red pulp, the splenic nodules being free of any but an occasional single cell. The nodules were composed mostly of small lymphocytes; only a few reticulum cells were seen, and there were no secondary centers or cell mitoses. A few cells containing a brown granular pigment were observed with the hematoxylin-eosin stain.

Within the pulp there were a few megakaryocytes but no indication of extramedullary hematopoiesis, which is so common in the spleen of aging mice. Mast cells mixed with lymphocytes and infrequent neutrophils crowded the

pulp. The cells that were considered neoplastic showed varying degrees of granulation, some cells containing a few granules taking a faint reddish violet stain with toluidine blue while other cells were so densely packed with granules stained a deep purple as to obscure the nucleus (fig. 2). In comparison with the normal (fig. 3), the neoplastic cells were usually larger, and when stained with toluidine blue the nucleus was more often visible, probably because the cytoplasm was not so packed with granules. The neoplastic cells were also compared with cells in the spleen of a full-term fetus which contained fewer granules than did the neoplastic cells (fig. 4). No forms were seen with the lobed or double nuclei which have often been interpreted as stages of amitotic division, and no mitoses were seen in cells which could be identified as belonging to possible precursor, nongranulated cells. The neoplastic cells often appeared to be crowded around the margin of vascular spaces in the spleen, and occasional mast cells were found, along with erythrocytes and leukocytes, within the lumina of vessels. The mast cells also appeared lined up and concentrated along the trabeculae. Cytoplasmic inclusions similar to those described by Bloom (*4*) in mastocytomas of the dog were found, but no crystalloids such as he reported were observed with the stains used. The inclusions were of two types, one eosinophilic and finely granular, and a second more common type consisting of a rounded basophilic body. These inclusions were often surrounded by unstained halos, and they resembled, as Bloom pointed out, the "birds-eye" inclusions described in carcinoma. Bloom also suggested the possibility that they may be virus inclusion bodies, or products of degenerative change in the cytoplasm. In many of the neoplastic cells, a number of these bodies were present; and it is our impression that they indicate a process of degeneration during which the uniform, fine, basophilic granules of the normal cell become fused into larger masses. Iron stains revealed no increase in iron-positive pigment in the spleen.

Lungs.—The mast cells were found grouped together, often surrounding a pulmonary vessel, and scattered within the walls of the alveoli and beneath the pleura. The neoplastic cells in the lung appeared to be less well preserved than those in the spleen, and basophilic cytoplasmic inclusions were more frequent (fig. 5). A notable histologic feature was the number of large macrophages usually found at the margin of mast-cell accumulations (fig. 6).

These phagocytes contained erythrocytes, iron-positive substances identified with the prussian-blue reaction, dark brown granules, occasional crystals, and rare granules staining metachromatically with toluidine blue, or bright red with fuchsin. Apparently, repeated hemorrhages had occurred, and the erythrocytes and products of hemoglobin destruction were ingested by the macrophages. Also phagocytized by these cells were mast-cell granules, which were either extruded from the cell or set free when the cell disintegrated.

Kidney.—Mast cells were located within the glomerular tufts and between the collecting tubules. They appeared to be better preserved than the cells of the spleen and lung. A few macrophages with iron-positive pigment were present in the kidney, but not in relation to aggregations of mast cells. The kidney showed an early lesion, with necrosis of the tip of the papilla, such as occurs frequently in certain strains of mice (Dunn (*14*)). A few cells of the proximal convoluted tubules also contained iron-positive granules, a condition which often accompanies this type of renal disease.

Duodenum.—Essentially normal.

Blood vessels.—Mast cells were found in blood vessels in the spleen but not elsewhere.

Adrenal and surrounding fat tissue.—There were a few scattered mast cells. No other organs or tissues were saved for histologic study.

Case 2

Case 2 was an LAF_1 female 24 months of age. At autopsy the kidneys appeared normal except for a small spot on one, which probably represented a mast-cell accumulation. One lung tumor was present.

Spleen.—In comparison with the normal, mast cells were considerably increased in number in the red pulp of the spleen, although they were not so numerous as in case 1. It was not possible to distinguish with certainty neoplastic cells from mast cells normally found at this site, and for this reason the actual degree of the neoplastic process could not be determined. Large spherical masses of basophilic substance (Bloom's cytoplasmic inclusions) or multiple smaller masses were frequent in the neoplastic cells, and in accumulations of these cells there were many containing relatively few and lightly stained granules.

Kidney.—One large aggregation of mast cells was found in the cortex, and a few were scattered singly in other cortical areas. The cells infiltrated the interstitial tissue in large numbers, and a few appeared in the glomeruli.

Duodenum.—It was doubtful whether there had been a neoplastic increase, but mast cells were unusually numerous in the submucosal connective tissue.

Lung.—Mast cells appeared singly and in small numbers in the alveolar walls. An adenomatous pulmonary tumor

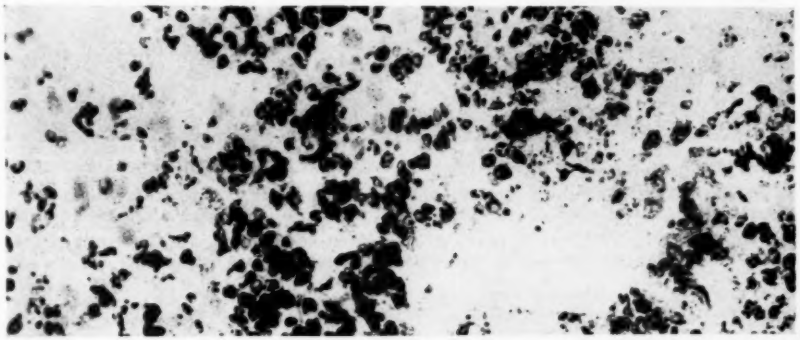

Figure 1.—Spleen, case 1, showing neoplastic mast cells accumulated in red pulp. The clear space represents splenic nodule. Toluidine blue. × 200.

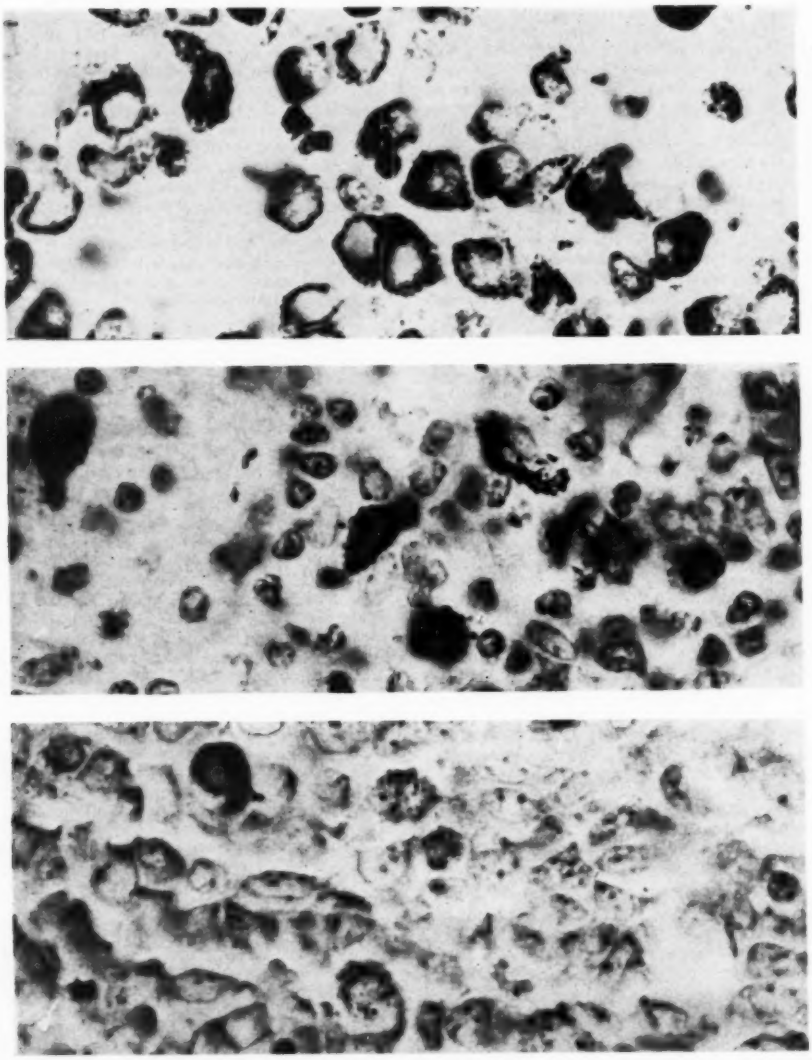

FIGURE 2 (top).—Spleen, case 1, showing neoplastic mast cells. Toluidine blue. × 1000.

FIGURE 3 (center).—Spleen of female strain A mouse, 7 months of age. Mast cells are abundant; nucleus is less evident; and cells are more packed with granules of uniform size than in figure 2. Toluidine blue. × 1000.

FIGURE 4 (bottom).—Spleen of fetus of strain A, taken on day of expected delivery. Three mast cells containing a few granules are seen in the center. Toluidine blue. × 1000.

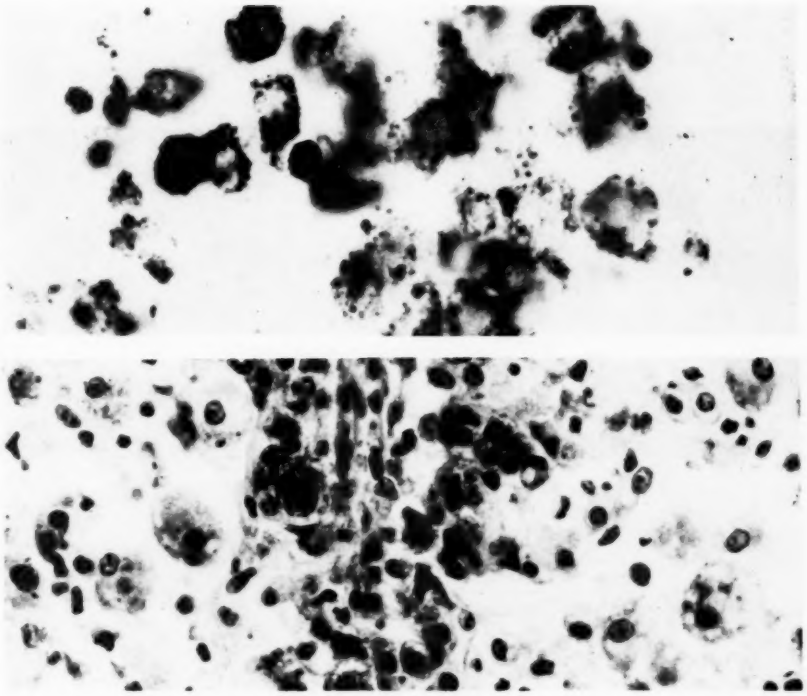

FIGURE 5 (top).—Lung, case 1, showing large cytoplasmic inclusion in the neoplastic cell in center. Variation in number and size of granules within cells. Toluidine blue. × 1000.

FIGURE 6.—Lung, case 1. Neoplastic mast cells with granular cytoplasm are concentrated about a small vessel. Large phagocytic cells with ingested pigment are seen at margins of mast-cell accumulations. Hematoxylin and eosin. × 500.

included in the section was free of these cells, and they were not increased at the margin.

Adrenal.—Mast cells were present and especially numerous in areas of the cortex where spindle cells replaced the usual vacuolated, lipid-containing cells. It was doubtful, however, that these cells were neoplastic.

Blood vessels.—A few mast cells were free in the vessels of the kidney.

CASE 3

This was an ALF_1 female 22 months of age. At autopsy the kidney appeared normal. The right inguinal lymph node measured 8.0 mm. in diameter. One lung tumor was present.

Inguinal lymph node.—This was the only site in which neoplastic mast cells occurred. Microscopically, the cells appeared most numerous in the medulla and the marginal sinus, the lymph follicles here, as in the spleen, being free of them. Distinct linear arrangements of the mast cells were found in the marginal sinus (fig. 7).

No neoplastic accumulations were found in any other organ available for study. The number in the spleen was well within the normal range, but a few of the mast cells contained cytoplasmic bodies. No mast cells were found in the kidney or lung.

CASE 4

Case 4 was a LA-ABC male 18 months of age. At autopsy there were tiny pale areas on the kidney surface, later identified as mast-cell accumulations. The liver was pale.

Spleen.—The number of mast cells in the spleen was not excessively increased, and it was not possible to determine what percentage of these were neoplastic.

Kidney.—Mast-cell aggregations were found at several different sites in the kidney cortex. They appeared within the intertubular tissue and often appeared concentrated at the margin of larger vessels. Mast cells were seen infrequently in the glomeruli.

Duodenum.—Numerous mast cells were found infiltrating the submucosal connective tissue, the lamina propria of the villi, and the interstitial tissue of Brunner's glands. Occasionally they were grouped together in masses formed entirely of this cell type, showing varying degrees of granulation (figs. 8–9), or they appeared in rows, as seen in the lymph node in case 3.

Liver.—This was the only case in which a section of liver was included. Amyloid was present, and mast cells were scattered singly throughout the liver sinuses.

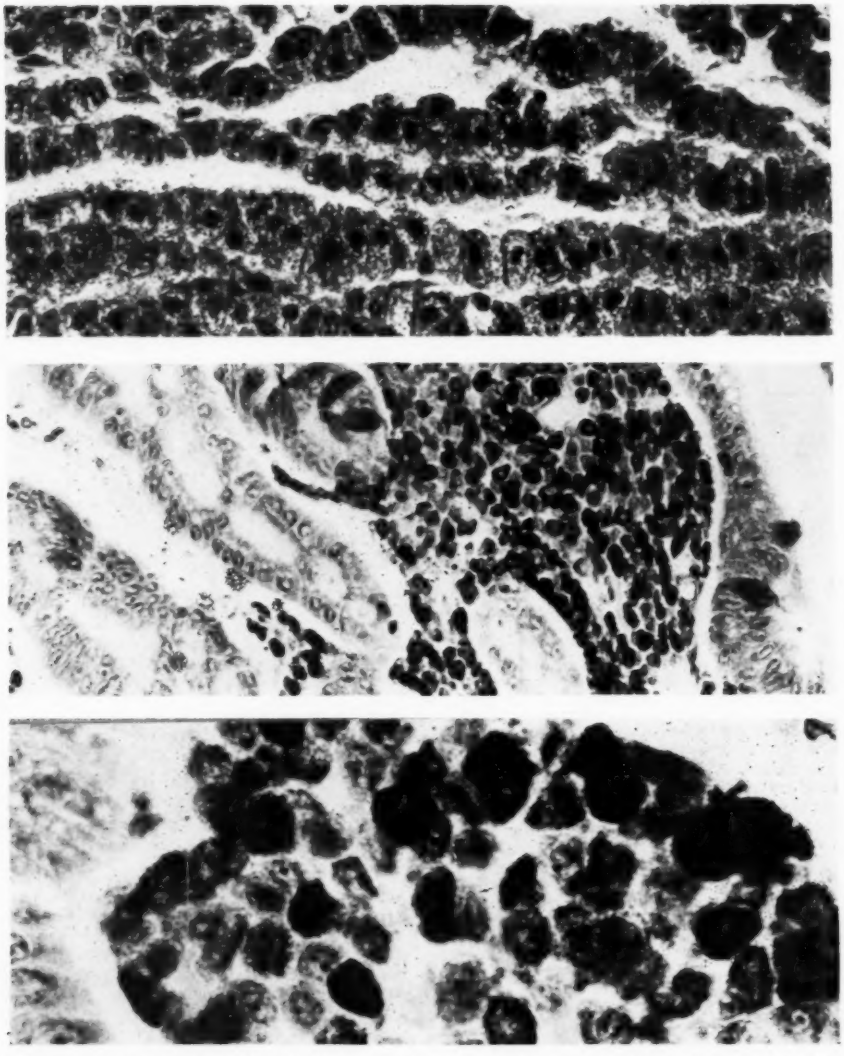

FIGURE 7.—Lymph node, case 3.—Neoplastic mast cells arranged in rows. Hematoxylin and eosin. × 500.

FIGURE 8.—Duodenum, case 4. Aggregation of neoplastic mast cells in lamina propria. Toluidine blue. × 300.

FIGURE 9.—Duodenum, case 4. Same field as in figure 8, with higher magnification. Observe variation in number and size of granules in neoplastic cells. Toluidine blue. × 1000.

CASE 5

Case 5 was an ALF_1 male 24 months of age. At autopsy the kidneys appeared normal. Two lung tumors were present.

Spleen.—Mast cells having neoplastic characteristics were very abundant and more numerous than those seen in normal spleens. They were often lined up along the walls of blood vessels.

Duodenum.—Mast cells appearing singly had infiltrated the muscle coat of the pylorus.

No other organs showed an unusual number of mast cells, and in this case a diagnosis of neoplasia cannot be definitely established.

MAST CELLS IN THE TISSUES OF MICE OF INBRED STRAINS

A few observations that are recorded here have been made on the occurrence of mast cells in mice of various inbred strains. The tissues were fixed in a saturated solution of basic lead acetate for several days followed by 10 percent formalin, or in Tellyesniczky's fixative. After either fixative, the tissues were embedded in paraffin, sectioned, and stained with toluidine blue. Basic lead acetate gave good preservation of granules, which usually appeared as distinct globular bodies. Tellyesniczky's fixative showed the granules less distinctly, but there was no difficulty in identifying the mast cell, and other cell elements could be stained by other methods after this fixative.

It was discovered that the number of mast cells in the spleen showed a definite strain variation (table 3). Mast cells were counted in a single longitudinal section of the spleen. In old strain A mice, these cells were so numerous that individual cells were indistinguishable, and only an estimate of the number could be made. The spleens of strain A mice at all ages were characterized by a large number of mast cells in the pulp, which appeared to increase with advancing age and to be slightly more numerous in the male. In the strain C3H and strain dba mice a moderate number of mast cells from 5 to 125 were present in the spleen, but an increase with age was not observed. Strain C57 black showed a slightly higher number, while the 5 strain I spleens examined showed the lowest number of any strain, averaging only 1 per longitudinal section. The difference in the number of mast cells in the spleens of strain A and strain I mice was also demonstrated by making smears of spleens and by staining them with Wright's stain. Only a few mast cells were found in the smear from a strain I mouse, and there appeared to be no scattering of granules in this preparation; whereas in the smear from a strain A mouse, the mast cells were extremely numerous, and there were many free basophilic granules seen throughout the smear. The number of mast cells in the spleens of strain L females was low, yet the hybrids of strains L and A showed a number above any of the other strains examined except strain A. The number in these hybrids, however, was still far below the parent strain A.

TABLE 3.—*Mast cells in spleens in various strains of mice*

Strain	Age of mice	Number	Sex	Mast-cell count [1]	
				Range	Average
A	6 weeks	5	M	123–389	268
	do	5	F	166–462	370
	3 months	5	M	600–1,100	928
	do	4	F	266–524	391
	7–13 months	11	M	1,000 above 2,000	[2]
	7–12 months	7	F	660 above 2,000	[2]
	15 months	2	F	1,500 above 2,000	[4]
C3H	6 weeks	5	M	13–61	41
	do	5	F	5–44	22
	3 months	4	F	5–32	13
	8–13 months	3	F	2–14	8
dba	6 weeks	5	M	1–3	2
	13–15 months	3	F	30–66–125	77
	15–19 months	2	M	63–68	65
L	19–21 months	8	F	8–24	16
	20–21 months	3	M	48–382	255
C57Bl	6 weeks	5	M	20–90	42
I	4–6 months	7	M	0	0
	9 months	5	F	0–3	1
Hybrid ALF_1	18–20 months	6	M	177–900	402
	18–22 months	8	F	59–325	192

[1] Number of cells per single longitudinal section.
[2] Above 2,000.
[3] About 1,500.
[4] About 2,000.

It would be interesting to know whether the total number of tissue mast cells are correlated with the number in the spleen. A determination of the total number in other tissues could only be approximated and would be very laborious if the careful method used by Bates (*15*) for the rat was followed. Sections have been made of other organs and subcutaneous tissue from various strains of mice. No increase in numbers in strain A mice was evident on inspection. Other peculiarities in strain A mice have been reported, namely, a high incidence of mammary tumors in breeding females (Bittner and Little (*16*)), a high incidence of pulmonary tumors (Heston (*17*)); frequency of primary amyloidosis and renal disease (Dunn (*14*)); and nephritis developing after administration of urethane (Dunn and Larsen (*18*)); but no causative relationship between these conditions and the

splenic mast cells can be suggested with our present limited understanding of these conditions.

Mast cells were found in other organs and tissues in the mice examined in about the following degree: frequently in the subcutaneous tissue, the tongue, uterine wall, connective-tissue and nerve-fiber sheaths; irregularly in the spleen, lymph nodes, and thymus; absent (for the strains examined) in the normal liver, kidney, heart, and lungs. They were found, however, in the kidneys of strain A mice in which the characteristic renal disease was noted. Here they were found in areas of fibrosis and atrophy, and the presence of mast cells under this condition must be explained as due either to transport by the blood stream or development in situ from a nongranulated, unrecognized, precursor cell. A few mast cells were usually found in the adrenal, and they appeared to be increased in areas of the cortex, frequently seen in mice, where fusiform, nonlipid-containing cells replaced the usual vacuolated, cortical cell.

In the fully developed fetuses removed from a strain A mouse just before delivery, numerous mast cells were seen in the subcutaneous tissues, and mitoses were not infrequent. Mast cells were also frequent in the spleen (fig. 4). The C3H fetus and the strain I fetus also contained numerous mast cells in the spleen as well as in the subcutaneous areas, and mitoses were found. Strain differences in the number of mast cells in the spleen, therefore, appear to develop as the animals age.

DISCUSSION

The usual objections may be raised concerning the neoplastic nature of these cellular accumulations, but the evidence points to a neoplastic rather than a nonneoplastic reaction. Considerable variation from the normal was seen in individual cells, which tended to be larger, and the contained granules varied greatly in number, size, and intensity of staining. The massive accumulations, often at sites in which mast cells are not normally found, and the absence of any common etiologic condition which could be recognized in these cases indicate a neoplastic proliferation and infiltration rather than an inflammatory reaction. Moreover, our ignorance as to any agent or condition to which a mouse is normally exposed and which is able to cause a proliferation of these cells, makes neoplasia the readiest explanation for the process. The disease probably progressed slowly since mitoses were few, and early death of the animals did not result.

The genesis of the mast cell is not yet agreed upon. It has been argued that the paucity of mitotic figures makes it improbable that mitotic division can account for the great local increase under various conditions. Amitotic division as a mode of reproduction for the tissue mast cell has been accepted by many authors, among them Michels (*1*), Bloom (*4*), Sabrazès and Lafon (*5*); and for the basophilic leukocyte by Doan and Reinhart (*2*). Lillie (*3*) and Twort and Twort (*7*) report that no mitoses were seen in the cases of mast-cell neoplasm studied by them. Lignac (*9*) makes no statement whether mitoses were present. Bates (*15*), studying mast cells in the subcutaneous tissue of the rat, reported that no mitoses were seen. Cramer and Simpson (*8*) saw no mitotic figures in mast cells and did not believe that the increase in their number after methylcholanthrene application could be attributed to the proliferation of the few originally present in these areas. Bloom, Michels, and Sabrazès and Lafon have also reported mitotic as well as amitotic division. Since we have seen cells containing unmistakable metachromatic granules showing mitoses in both fetal and neoplastic tissue, it is our opinion that the mast-cell increase can be explained by this process; and there is only presumptive evidence for an amitotic division, although development from a nongranulated precursor type of cell is suggested in some of these growths and also by the finding of these cells in unusual sites under pathologic conditions. The mitoses are extremely rare; but since we have no evidence that mast cells carry out any function during which they are used up or destroyed and since this cell always remains relatively rare under normal conditions, replenishment should be infrequent. This lack of proliferative activity may also account for the rarity of a neoplastic change.

Recently Lison (*19*) and his collaborators have advanced evidence that the mast-cell granule is related to heparin. In connection with this, it is interesting to note that several reported mast-cell neoplasms have been characterized by considerable evidence of hemorrhage and blood destruction. Lignac (*9*) observed that the reticular cells in his cases contained large masses of disintegrated pigment; but this condition he ascribed to the toxic effect of the benzol, since it was found generally in mice exposed to this agent. One of his mice also had a hemorrhagic ascites. Twort and Twort (*7*) described a central mass of tumor in the spleen, with two lateral areas of blood which contained lysed patches of tumor cells. The tumor edge was on the whole sharply defined from the hemorrhagic tissue. They also considered it noteworthy that many of the splenic tumor cells were full of red blood corpuscles, while no intracellular blood cells were seen in tumor cells in the liver. If this observation is accurate and if the authors have not mistaken mononuclear phagocytic cells which have ingested both erythrocytes and mast-cell granules for true tumor cells, it is evidence, although the only evidence, found in these reports of phagocytic activity in the

tissue mast cell. In the mast-cell tumor of the horse described by Sabrazès and Lafon (*5*), it was noted as remarkable that in certain regions of the tumor there were large hemorrhagic foci, veritable hematomas, containing numerous "hematomacrophages" loaded with pigment and some hematoidin crystals. The authors believed that granules derived from hemoglobin disintegration and metachromatic granules were present in the same cell, and that all the stages between the "hematomacrophage" and the mast cells could be recognized. They also saw stages which convinced them that mast cells might be derived from lymphocytic elements, muscle or fibroblast cells. Neither Bloom (*4*), Lillie (*3*), nor Doan and Reinhart (*2*) reported any unusual hemorrhagic reaction in their cases. In case 1 the hemorrhagic reaction in the lungs is remarkable, and it would be interesting to interpret it as being due to a heparin effect exerted by the mast cell granules. On the other hand, the absence of hemorrhages in the spleen and kidney of this case and in all organs of the other four cases throws doubt on such an interpretation. We observed, as did Sabrazès and Lafon, that macrophage cells containing both mast cell granules and blood pigment could be found. Although the neoplastic condition distorts the physiologic behavior as well as the morphologic appearance of a cell and although the mere presence of heparin would not necessarily provoke a hemorrhage, still the evidence so far available from these neoplasms does not offer much support to the theory of the heparin-bearing function of the mast cells. A certain amount of hemorrhage is not uncommon in the vicinity of tumors, and a larger number of cases needs to be studied. Unfortunately, this tumor has not been carried in transplant, although Lignac made unsuccessful attempts with each of his mice. The lack of success may have been due to the use of heterogeneous mice.

The question of a relation of the mast cell to cancer has been considered in the paper by Cramer and Simpson (*8*). They noted a remarkable accumulation in the subcutis after repeated paintings with methylcholanthrene. The mast cells, however, did not occur within the developing tumors, and they were even more frequent in resistant mice in which no tumors occurred. From this, the authors suggested that the mast cell may have a defensive role in neoplasia. Twort and Twort (*7*) considered the mast-cell increase after tar painting as roughly proportional to the amount of subepithelial inflammation present. Conditions reported in the human skin also suggest a relation to inflammatory conditions, and this appears to us a more reasonable explanation than any direct antineoplastic effect. Sylvén (*20*) studied histologically the margins of mesenchymal tumors and observed that mast cells accumulated in tissues adjacent to rapidly growing, infiltrating, malignant tumors. His suggestion is that a substance, probably heparin, within the cell actually assists infiltrative growth. Such opposite views are difficult to reconcile, and the problem is further complicated when this cell can itself undergo a neoplastic change. More facts are required before a conclusion in regard to the function of the mast cell can be reached. This study of mast cells under neoplastic conditions may have a bearing on some uncertainties about the origin and function of this cell.

NOTE.—After this paper was ready for publication, another previously unrecognized mast-cell neoplasm was diagnosed in a hybrid strain A backcross mouse 21½ months old. The neoplasm measured 8 mm. in diameter and had developed below the mucosa in the wall of the stomach. Giemsa's method and toluidine blue readily disclosed the nature of the process. Many cells were detached from the main tumor mass and had infiltrated into the lamina propria and the muscle wall. Histologic material from this group of aged hybrid mice is now being examined for possible mast-cell neoplasms. Many cases of this lesion may be missed because of an inadequate fixation and staining method to preserve and show the mast-cell granulations.

SUMMARY

Gross and microscopic observations of five cases of mast-cell neoplasia occurring in reciprocal F_1 hybrid mice of strains A and L are reported.

Observations on the occurrence of mast cells in single longitudinal sections of the spleen in various inbred strains of mice are recorded. There appears to be a considerable difference in the number of these cells, which was highest in old strain A males.

The possible bearing of these observations on theories regarding the origin and function of the mast cell and the relation of these cells to the process of neoplasia are discussed.

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